

TRANSMIT

DESIGNA

OFFICE (DO/EO/US)

CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
BOH6278P0020US

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

Unassigned 09/937820

INTERNATIONAL APPLICATION NO.

PCT/EP00/02727

INTERNATIONAL FILING DATE

28 MARCH 00

PRIORITY DATE CLAIMED

29 MARCH 99

TITLE OF INVENTION

Methods for the Identification and Validation of Functional Intracellular Targets with Intramers or *in vivo* Selection

APPLICANT(S) FOR DO/EO/US

Michael Blind, Michael Fainulok and Waldemar Kolanus

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
- ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
- ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
- ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). (**Unsigned**)
- ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
- ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
- ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

International Search Report

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

INTERNATIONAL APPLICATION NO.

ATTORNEY'S DOCKET NUMBER

09/19/97 820

PCT/EP00/02727

BOH6278P0020US

24. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **\$1000.00**
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **\$860.00**
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **\$710.00**
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **\$690.00**
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **\$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =**CALCULATIONS PTO USE ONLY****\$860.00**Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).**\$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	128 - 20 =	108	x \$18.00
Independent claims	8 - 3 =	5	x \$80.00
Multiple Dependent Claims (check if applicable).			<input checked="" type="checkbox"/>

\$1,944.00**\$400.00****\$270.00****TOTAL OF ABOVE CALCULATIONS =****\$3,474.00**☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.**\$1,737.00****SUBTOTAL =****\$1,737.00**Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).**\$0.00****TOTAL NATIONAL FEE =****\$1,737.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☐**\$0.00****TOTAL FEES ENCLOSED =****\$1,737.00**

Amount to be:	\$
refunded	
charged	\$

- ☒ A check in the amount of **\$1,737.00** to cover the above fees is enclosed.
- ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **04-1644** A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

MULLER, Lisa V.
ROCKEY, MILNAMOW & KATZ, LTD.
Two Prudential Plaza
180 N. Stetson Ave., Suite 4700
Chicago, Illinois 60601
(312) 616-5400

SIGNATURE

MUELLER, Lisa V.

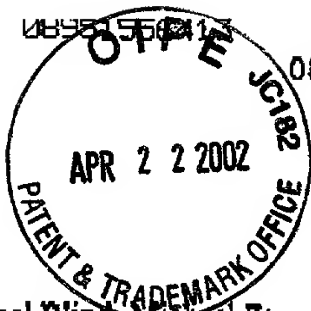
NAME

38,978

REGISTRATION NUMBER

September 26, 2001

DATE



Applicant or Patentee: Michael Blind, Michael Famulok, Waldemar Kolanus Atty Docket No. BOH6278P0020US
Serial or Patent No.: _____
Filed or Issued: _____
For: Methods for the Identification and Validation of Functional Intracellular Targets with Intramers or in vivo Selection

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. 1.9(d) AND 1.27(b) - INDEPENDENT INVENTOR)

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 C.F.R. 1.9(c) for purposes of paying reduced fees to the United States Patent and Trademark Office with regard to the invention entitled: _____ described in:

- ☒ the specification filed herewith.
☐ Application Serial No. _____, filed _____
☐ Patent No. _____, issued _____

I have not assigned, granted, conveyed, or licensed, and am under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 C.F.R. 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. 1.9(d) or a nonprofit organization under 37 C.F.R. 1.9(e).

Each person, concern, or organization to which I have assigned, granted, conveyed, or licensed, or am under an obligation under contract or under law to assign, grant, convey, or license any rights in the invention, is listed below:

- ☐ no such person, concern, or organization.
☐ person, concerns, or organizations listed below.*

*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. 1.27)

FULL NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

x
BLIND, Michael

Signature of Inventor
25/09/01
Date

x
FAMULOK, Michael

Signature of Inventor
25/09/01
Date

x
KOLANUS, Waldemar

Signature of Inventor
16-10-2001
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Michael Blind et al.)	Attorney Docket No. BOH6278P0020US
)	
Serial No.:	Unassigned)	Group Art Unit: Unassigned
)	
Filed:	September 26, 2001)	Examiner: Unassigned
)	
For:	Methods for the Identification)	
	and Validation of Functional)	
	Intracellular Targets with)	
	Intramers or <i>in vivo</i> Selection)	

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Please cancel claims 1-17 and replace them with claims 18-35 as follows:

18. A method for the identification of an intramer capable of binding to and modifying the function of a functional intracellular target, comprising:
- a) preparing a candidate intramer mixture of nucleic acids;
 - b) contacting the candidate intramer mixture of nucleic acids with the intracellular target or part thereof whereby the target or the part thereof is not known to bind RNA prior to step (b);
 - c) selecting and isolating nucleic acids having an increased affinity to said target reactive to the candidate intramer mixture;
 - d) reverse transcribing, if the candidate intramer mixture comprises RNAs, and amplifying the nucleic acids obtained in step (c);
 - e) optionally repeating steps (b) to (d);
 - f) isolating and sequencing the clones (intramers) obtained in step (e);
 - g) testing whether the expression product of the insert of the clone in step (f) binds to and affects the function of the intracellular target *in vivo*.

19. The method of claim 18, wherein step (g) is carried out by using a cytoplasmic expression system.

20. The method of claims 18 or 19 further comprising:

h) mapping of the binding site of the intramer to said target.

21. A method for the identification of a functional intracellular target which is associated with a particular phenotype and the corresponding intramer capable of binding to and modifying the function of said target, comprising:

a) preparing a candidate intramer mixture of nucleic acids;

b) cloning the candidate intramer mixture of nucleic acids under the control of a suitable promoter in a vector optionally containing a selectable marker;

c) introducing the vector obtained in step (b) into a reporter cell line allowing the positive or negative phenotype selection;

d) selecting cells with an altered phenotype; and

e) determining the sequence of the nucleic acid inserted in the vector of step (b) (intramer) and the compound to which it binds.

22. The method of claims 18, 19, 20 or 21, wherein said candidate intramer mixture of nucleic acids comprises single stranded nucleic acids.

23. The method of claim 22, wherein the single stranded nucleic acid is a RNA.

24. The method of claims 18, 19, 20, 21, 22 or 23, wherein the functional intracellular target is an integrin.

25. The method of claims 21, 22, 23 or 24, wherein the reporter cell line of step (c) allows negative selection.

26. The method of claim 25, wherein the reporter cell line contains a vector

comprising a selectable marker and a reporter gene encoding a toxin under the control of an inducible promotor and wherein only cells will survive expressing the vector of step (b) which expresses a nucleic acid (intramer) inhibiting a compound which is required for the activation of the promotor controlling the toxin gene, and wherein in step (d) the surviving cells are selected.

27. The method of claims 21, 22, 23, 24, 25 or 26, wherein the candidate intramer mixture of nucleic acids of the vector of step (b) is under control of a Pol III promoter.

28. The method of claims 26 or 27, wherein the toxin gene is HSV-thymidine-kinase.

29. The method of claims 18, 19, 20, 21, 22, 23, 24, 25 or 26, wherein the promotor controlling the toxin gene is an IL-2 promoter.

30. A host cell containing one or more vectors having the candidate intramer mixture recited in claims 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29 as insertions and/or vector of claim 26.

31. A pharmaceutical composition containing a nucleic acid (intramer) which is capable of modifying the function of the functional intracellular target to which it binds and which is identified according to the method of claims 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29, optionally in combination with a suitable pharmaceutical carrier.

32. A pharmaceutical composition containing a compound which is capable of modifying the function of the functional intracellular target identified by the method of claims 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29.

33. Use of the nucleic acid identified by the method of claims 18, 19, 20, 21, 22,

23, 24, 25, 26, 27, 28 or 29.

34. Use of a compound which is capable of modifying the functional intracellular target identified by the method of claims 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30.

35. Kit useful for the diagnoses of diseases associated with abnormal concentrations and/or modifications of the intracellular target to which a nucleic acid identified by the method of claims 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29.

Applicants submit that the above-identified application is now in condition for allowance. If any fees are incurred as a result of the filing of this paper, authorization is given to charge Deposit Account No. 04-1644.

Respectfully submitted,

By


Lisa V. Mueller, Reg. No. 38,978

ROCKEY, MILNAMOW & KATZ, LTD.
Two Prudential Plaza
180 N. Stetson Ave., Suite 4700
Chicago, Illinois 60601
312-616-5400

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this Preliminary Amendment and any other documents referred to as enclosed herein, are being deposited in an envelope with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated below and addressed: Commissioner For Patent, Washington, D.C. 20231.

EL904822871US
Express Mail Label No.

September 26, 2001
Date of Deposit

Clarence Townsend
Typed/Printed Name of Person Mailing Correspondence

Clarence Townsend
Signature of Person Mailing Correspondence

12/PRTS

09/937820
JC05 Rec'd PCT/PTO 26 SEP 2007

Methods for the identification and validation of functional
intracellular targets with intramers or in vivo selection

The present invention relates to the intracellular application of functional nucleic acids, termed "intramers", which affect the biological function of an intracellular component (e.g. inhibit its function within the context of a living cell by specifically complexing the component). It could be shown that a) intramers can be placed into a functional context inside cells regardless whether the target naturally binds nucleic acids or not; b) intramers can convey effects to intracellular sites in which a nucleic acid normally is not found. This method is useful to enable the elucidation of the biological role of a wide variety of intracellular components. The present invention furthermore relates to methods for the expression of randomized nucleic acid libraries inside cells to identify functional intramers which alter the phenotype of the cell in which they are expressed. This method is, e.g. useful for the identification of an intracellular target as functionally responsible for or involved in a certain cellular phenotype without any prior knowledge on the biological function of the component. Finally, the present invention relates to pharmaceutical compositions comprising the "intramers" identified by the methods of the present invention or, alternatively, compounds showing the same or a similar target specificity and/or effect, e.g. for gene therapy.

In recent years significant progress has been achieved in identifying the complete set of genetic information of various organisms. The genomes of procaryotes such as E.coli or eucaryotes such as S.cerevisiae (Goffeau et al., Science 274 (1996), 546-567) and C. elegans (The C. elegans Sequencing Consortium, Science 282 (1998), 2012-2018) were completely sequenced. It is estimated that the complete human

09/937820-042000

genome will be known in 3-5 years. The challenge one has to face after these achievements, however, is to link a function to every single gene. For example, a major goal is to identify those gene products as potential drug targets, which play key roles in the complex network of protein interactions that ultimately lead to diseases (see e.g. Friedrich, Nat. Biotechnol. 14 (1996), 1234-7). To identify these key molecules among the 100000 human genes the number of possible candidate molecules is defined by comparing the protein composition of different developmental- or disease states of a cell by differential gene expression. The actual status of the protein composition of a cell can be obtained by analyzing the mRNA of the cell (for reviews of the various methods see Wan et al., Nat. Biotechnol. 14 (1996), 1685-1691). This method, however, only provides an indirect and not very accurate measure of the cell's current proteome status because the mRNA may long be degraded while the protein is still present, or a high amount of mRNA has been transcribed but cannot be translated for some reasons. It is also not possible to cover posttranslational modifications of the expressed proteins. An alternative way to cover the proteome-status of a cell via "proteomics" is 2D gel electrophoresis. Using this method one can attempt to cover the composition of all expressed proteins in a cell at a given time in the form of separate spots on a gel whereby each spot corresponds to an individual protein. By comparing different gels, different proteins or amounts of proteins can be identified. However, this method currently allows to identify at best about 20% of expressed proteins in a higher eucaryote. The detection of low copy number proteins is problematic. In addition, both methods only allow to look at the presence or absence of gene products in a particular developmental or disease state. They do not allow to state whether or not the identified differences in the expression pattern cause the cellular status or are just a consequence thereof.

The direct analysis of the function a certain protein complex, or one of its subunits or domains exhibits in a cellular process can be achieved via the genetic "knock-out" (homologous recombination, antisense technologies), or via

overexpression or mutation of the protein. This, however, always results in an alteration of the genetic information of an organism which makes the interpretation of the results difficult. For example, the knock-out of a gene does not allow to draw conclusions which part or domain of a protein is important for its function. In addition, the expression of other genes is affected in most cases (for a review see: Proteome Research: New Frontiers in Functional Genomics, Springer-Verlag (1997), 1-30).

There is thus a high demand for specific intracellular inhibitors or modulators which can be applied in a certain time-window and which enable the analysis of a certain unaltered protein within its natural expression status [(Spencer et al., Science 262 (1993), 1019-1024; Huang & Schreiber, Proc. Natl. Acad. Sci. USA 94 (1997), 13396-13401]. Most inhibitors or modulators currently known are based upon membrane permeable small organic molecules which often are of limited specificity and are available only for particular proteins. This limitation may be overcome by intracellular antibodies ("intrabodies"; see Richardson, Tibtech 13 (1995), 306-10) or peptide aptamers (see Colas et al. Nature 380 (1996) 548-550). The problem with intracellular antibodies, however, is, for example, to adjust the extracellular antibody to the reductive intracellular compartment. Processes such as dimerization of heavy- and light chain, and the stabilization by disulfide bonds or glycosylation occur intracellularly with very low efficiency or not at all and have to be compensated by costly design of the polypeptide chains.

Functional nucleic acids acting within the context of a living cell can help to fulfill the demand for specific intracellular inhibitors or modulators which can be applied in a certain time-window and which enable the analysis of a certain unaltered protein within its natural expression status.

Functional nucleic acids are a single stranded DNA (ssDNA) or RNA (ssRNA) or chemically modified nucleic acids

(ssNAmod), double stranded DNA (dsDNA) or RNA (dsRNA) or chemically modified forms thereof (dsNAmod) which are able to bind, modulate or catalytically modify an intracellular target thereby affecting its biological function. A functional nucleic acid can be identified for example through in vitro selection or "SELEX" (systematic evolution of ligands by exponential enrichment; see Tuerk and Gold, *Science* 249 (1990), 505-510; Ellington and Szostak, *Nature* 346 (1990) 818-822), or through ribozymes which can be activated allosterically (aptazymes; see Robertson and Ellington, *Nat. Biotechnol.* 17 (1999), 62-66). It is well established that functional nucleic acids, for example aptamers generated by in vitro selection, can bind to a wide variety of ligands ranging from small molecules and biological cofactors to natural and synthetic polymers including proteins, polysaccharides, glycoproteins, hormones, receptors and cell surfaces. In general, nucleic acids can be transferred into cells by methods commonly known to the expert such as lipofection, electroporation or by means of vectors which enable high rates of transcription, e.g. plasmids with RNA polymerase III promoters, T7 RNA polymerase/vaccinia virus based systems, or replication, e.g. Semliki forest virus.

Examples of small, intracellularly applied nucleic acids so far are limited to antisense nucleic acids, catalytic antisense nucleic acids such as hammerhead ribozymes (Birikh et al., *Eur. J. Biochem.* 245 (1997), 1-16; Bramlage et al., *Trends Biotechnol.* 16 (1998), 434-438) and nucleic acid ligands which bind via an aptameric mechanism to certain targets that are limited to naturally nucleic acid binding proteins, such as the Rev-protein from HIV-1 (Good et al., *Gene Ther.* 4 (1997), 45-54; Symensma et al., *J. Virol.* 70 (1996), 179-187), RNA polymerase II from yeast (Thomas et al., *J. Biol. Chem.* 272 (1997), 27980-27986), the SelB protein from *E. coli* (Klug et al., *Proc. Natl. Acad. Sci. USA* 94 (1997), 6676-6681). These examples, however, do not fulfill the demand for specific intracellular inhibitors or modulators which can routinely be applied in a certain time-window and which enable the analysis of a certain unaltered

cellular component such as a protein within its natural expression status. So far it has only been established that in vitro selected aptamers which bind to a naturally nucleic acid-binding protein, and are usually closely related to the corresponding natural nucleic acid sequence, still do so inside the cell. It has not yet been established, for example, that an aptamer can generally be applied to affect the biological function of intracellular targets, including, for example, hormones, biological cofactors, non-nucleic acid-binding proteins, biopolymers etc.

Thus, the technical problem underlying the present invention is to provide means for the identification and validation of intracellular targets including targets which naturally do not bind to nucleic acids and for the modification of the function of said target.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

The present invention is based on the unique insight that nucleic acid ligands can be targeted against practically any cellular component, and can be easily applied intracellularly whereby

- a) they still recognize their target in the intracellular environment;
- b) they can do so even with targets that are non-nucleic acid-binding by nature;
- c) they can localize their target in subcellular compartments such as the cytoplasmic face of the cell membrane where nucleic acids are normally not found;
- d) they can modulate the biological function of the target which allows conclusions with respect to its biological role; and
- e) they can alter the phenotype of the cell.

This enables the general application of intracellular functional nucleic acids for the validation of the function of intracellular targets, preferentially for the selection or validation of novel drug-targets. If desired, the

functional nucleic acid sequence can be placed within an expression cassette or other sequence context that may be useful for

- a) increasing the stability of the functional nucleic acid within the cellular compartment or providing signals, e.g. for correct termination of a functional RNA sequence that is expressed under the control of RNA polymerases; and
- b) providing additional sequence information that is required for the correct localization of the functional nucleic acid, e.g. to transfer the nucleic acid from the nucleus to the cytoplasm.

Once brought into the cell, these nucleic acids will exert their biological effects which can be studied. The method thus could significantly contribute to the field of "functional genomics".

Accordingly, in a first aspect the present invention relates to a method for the identification of an intramer capable of binding to and modifying the function of a functional intracellular target, comprising:

- a) preparing a candidate mixture of nucleic acids;
- b) contacting the candidate mixture of nucleic acids with the intracellular target or a part thereof;
- c) selecting and isolating nucleic acids having an increased affinity to said target relative to the candidate mixture;
- d) reverse transcribing, if the candidate mixture comprises RNAs and amplifying the nucleic acids obtained in step c);
- e) optionally repeating steps b) to d);
- f) isolating and sequencing the clones (intramers) obtained in step e); and
- g) testing whether the expression product of the insert of the clone obtained in step f) binds to and affects the function of the intracellular target in vivo.

Preferably, step g) is carried out by using a cytoplasmic expression system.

2004-07-02 09:45:50

In a more preferred embodiment the method of the present invention furthermore comprises

- h) mapping of the binding site of the aptamer to said target.

The present invention furthermore relates to methods for the expression of randomized nucleic acid libraries inside cells for the identification of functional intramers which alter the phenotype of the cell in which they are expressed. Based on the inventor's insight of the general applicability of functional nucleic acids to affect intracellular targets it is also feasible to express libraries of randomized RNA sequences inside cells. These complex libraries of randomized nucleic acids contain functional molecules which bind to cellular components such as enhancers or repressors of signal transduction pathways and modulate their biological function resulting in an altered phenotype of the cell. These molecules can be identified with appropriate selection methods. Modulation not necessarily involves mechanisms that rely on mere binding but can also, for example, involve catalytic processes, such as ribozymatic modification of an intracellular target.

Once a certain phenotype is observed, it is possible to identify the cellular target(s) responsible for the phenotypic change. This can be done by variations of different technologies such as the "three hybrid system (SenGupta et al., Proc. Natl. Acad. Sci. USA, 93 (1996), 8496-8501) or other methods described in the examples below. In general, it is possible to identify the cellular target by taking advantage of the interaction between the active RNA from the library with the target. This system is useful for the targeted identification of key molecules involved in a certain phenotype, preferentially a phenotype associated with a certain disease. It is superior to other systems currently available because it circumvents the need for screening a large number of potential candidates which have been identified indirectly, for example by proteomics, but rather identifies the key molecules in a direct phenotypic

selection process. The identified key molecules are potential drug targets; the nucleic acid modulators are not only target validators, but also potential drug-leads which could even be applied as drugs directly, for example in gene therapeutic approaches.

Accordingly, in a further aspect the present invention relates to a method for the identification of a functional intracellular target which is associated with a particular phenotype and the corresponding intramer capable of binding to and modifying the function of said target, comprising:

- a) preparing a candidate mixture of nucleic acids;
- b) cloning the candidate mixture of nucleic acids under the control of a suitable promotor in a vector, optionally containing a selectable marker;
- c) introducing the vector obtained in step b) (intramers) into a reporter cell line allowing positive or negative phenotypic selection;
- d) selecting cells with an altered phenotype; and
- e) determining the sequence of the nucleic acid inserted in the vector of step b) and the compound to which it binds.

Brief description of the Figures

A: Consecutive outline of Figures 1 - 5 (for Example 1)

Figure 1: Selection and characterisation of CD18cyt-specific RNA aptamers. **A**, Construction of the synthetic DNA pool and primary 46-mer amino acid sequence of the complete cytoplasmic domain of β 2-integrin (CD18cyt), immobilised to sepharose and used in the selection. **B**, Sequences and predicted secondary structures of individual aptamer clones D20, D28, D31, and D42. Nucleotides shown in bold-face are part of the randomised region. The aptamer region shaded in grey in clones D28 and D20 were shown in a damage selection experiment to be minimal requirements for retaining CD18cyt-binding capacity. The sequence D42 was used as a negative control; this sequence does not show any detectable binding to CD18cyt.

Figure 2: RNA aptamer expression system based on double infection with recombinant vaccinia viruses. **A**, Design of the T7-RNA expression cassette. **B**, Schematic representation of the vaccinia virus-based cytoplasmatic RNA aptamer expression system. **C**, Course of the expression of TR-encoded aptamer in vT7 coinfecting Jurkat E6 cells shown by a representative dot blot analysis for aptamer TR-D31 (right panel). Maximum levels of aptamer expression are seen 7 h post infection. Quantification (left panel) was done as follows: total cellular RNA was isolated, transferred to the blotting membrane and hybridized with 5'-³²P-labeled oligonucleotide complementary to the 3'- stem-loop structure present in all TR-constructs. For comparison and quantification *in vitro* transcribed aptamer TR-D20 was treated in the same way. **D**, Dot blot analysis and quantification of maximally expressed aptamer RNA. Each dot was quantified on a phosphor imager. Quantification was done as described for Figure 2c.

Figure 3: Gel mobility shift assays of endogenous CD18 contained in Jurkat E6 cell lysates bound to its cognate aptamer TR-D20. **A**. Gel shift experiment 1. Lane 1: free TR-D20 aptamer RNA; lane 2: shifted band obtained in presence of Jurkat E6 lysate and 25 μ M unspecific competitor tRNA; lane 3: no shift obtained with negative control sequence TR-D42; lane 4: same as lane 2 with 40 μ M unspecific competitor tRNA; lane 5: same as lane 3 with 40 μ M unspecific competitor tRNA; lane 6: specific supershifted band obtained in the presence of antibody MHM23 which recognizes the β 2-extracellular domain of LFA-1; lane 7: specific supershifted band obtained in the presence of antibody MEM170 which recognizes the extracellular domain of the β L-subunit of LFA-1. The band pattern may reflect aptamer/integrin complexes of different stoichiometry. **B**. Gel shift experiment 2 with additional controls. All gel shift experiments were performed in the presence of 30 μ M tRNA as a non-specific competitor. Lane 1: free TR-D20 aptamer RNA; lane 2: shifted band obtained in presence of Jurkat E6 lysate; lane 3: specific supershifted band obtained in the presence of

antibody MHM23; lane 4: no shifted aptamer obtained in the absence of Jurkat E6 cell lysate under conditions identical to those in lane 3. lane 5: specific supershifted band obtained in the presence of antibody MEM170; lane 6: no supershift obtained in the presence of antibody OKT3 directed against the T-cell receptor. The experimental conditions are otherwise the same as in lane 2; lane 7: no shifted aptamer obtained in the absence of Jurkat E6 cell lysate under conditions identical to those in lane 6.

Figure 4: Inhibition of PMA-stimulated cell adhesion as a function of aptamer expression. **A.** The CD18cyt binder TR-D20 reduces phorbol-ester activated Jurkat cell adhesion to ICAM-1. Jurkat E6 cells infected with recombinant vaccinia viruses were allowed to adhere to plastic dishes coated with a recombinant ICAM-1 chimera as described in example 1 section. Jurkat E6 cell adhesion to ICAM-1 was superinducible by PMA in the presence of several control viruses (vT7/vTR, wild-type vaccinia virus, vT7, vTR-D20). However, induction of CD18cyt-specific aptamer expression (vT7/vTR-D20, right panel) reduced PMA-stimulated Jurkat E6 cell adhesion, but not basal adhesion. These results were independently reproduced at least three times. Percentage reflects values normalized to stimulated vT7/vTR double infection which was set to 100%. **B.** Human peripheral blood mononuclear cells are good targets for protein overexpression by recombinant vaccinia viruses. Cytoplasmic immunoglobulin (cIg) fusions of cytohesin-1 subdomains were detected with the help of a FITC-conjugated anti-human Ig antibody preparation in permeabilised PBMC. Upper panel, left: control infection, no recombinant molecules expressed; upper panel, right: cIg-domains alone; lower panel, left: cIg-PH; lower panel, right: cIg-PH+c-domain. **C.** TR-D20 specifically reduces PMA-stimulated adhesion of PBMC to ICAM-1. Aptamers or control proteins were expressed in PBMC by recombinant vaccinia viruses as described above. PMA-stimulated adhesion of these cells to ICAM-1 was equivalent when the TR-D42 aptamer, cIg, or cIg-PH control proteins were expressed, but stimulated cell adhesion was

dramatically reduced following expression of the intact PH + c-domain of cytohesin-1 (cIg-PH+c) or of the TR-D20 aptamer.

Figure 5: Mapping of the binding site of aptamers TR-D20, TR-D28, TR-D31, and the negative control sequence TR-D42 on CD18cyt using synthetic biotinylated peptide fragments. All gel shifts were carried out in presence of 25 μ M streptavidine to enhance separation of shifted- versus non-shifted RNA. Gel shift experiments were performed in presence of 4 nM radiolabeled RNA and 40 μ M nonspecific tRNA competitor. Lane 1: free aptamer in the presence of 25 μ M streptavidine and 40 μ M unspecific competitor tRNA; lanes 2, 4, 6 or 3, 5, 7: same as lane 1 in presence of 25 μ M or 12.5 μ M, respectively, peptides A23 (lane 2, 3), B16 (lane 4, 5), and C17 (lane 6, 7).

B: Consecutive outline of Figures 6 - 12 (for Example 2)

Figure 6: Vectors for the phenotypic selection. **A** Scheme of important features of plasmid pP1; *CMV P*: promotor element: human cytomegalovirus immediate early promotor. Example of a constitutive promotor element that is active in a wide variety of cell types. Depending on cell lines, application, etc. other frequently used promotors or combinations of promoter elements (e.g. SV 40 early promotor, Rous sarcoma virus immediate early promotor etc.) can be used; *IL-2 P*: IL-2 promotor; *neo*: Selectable marker: commonly used resistance gene (aminoglycosid phosphotransferase) to select for cells harbouring the transfected plasmids via detoxification of aminoglycosid antibiotics; *hsv thymidine kinase*: gene for the herpes simplex virus thymidine kinase. **B** Scheme of important features of plasmid pP2; *CMV P*: see pP1; *Pol III P*: promotor element: RNA Polymerase III promotor; *vai random vai*: random nucleic acid library with the adenoviral VAI RNA sequences that permit the export of the functional nucleic acid into the cytoplasm; *OriP*: Episomal replication: The Epstein Barr virus (EBV) origin of replication and nuclear antigen (EBNA-1) allow the high copy episomal (extra chromosomal) replication and maintenance in primate and canine cell lines; *EBNA-1*: gene for the Epstein

Barr virus nuclear antigen1; *hygromycin*: Resistance against hygromycin can be used to co-select for cells that harbor two plasmids (e.g. an additional plasmid with the neo resistance)

Figure 7: Scheme for the functional selection against the induction of the interleukin 2 promotor in T-cells after stimulation with phorbol-12-myristate-13-acetate (PMA). The protein cascade which is shown schematically in this example leading from protein X via Y to Z (straight arrows) in a T-cell, ultimately results in the activation of the interleukin-2 promotor (IL-2P) of the reporter construct IL-2 P/hsv-thymidine kinase. Transcription and translation of the mRNA of hsv-thymidine kinase (mRNA HSV TK) results in the expression of Herpes simplex thymidine kinase (HSV TK) (dashed arrows represent gene expression). Constitutive transcription of the sequences of a nucleic acid library (vai/random/vai) under the control of an RNA polymerase III promotor (Pol III P) results in the formation of a randomized RNA library which is exported into the cytoplasm due to its adenoviral VAI sequence. As indicated in this figure, the expression of a non functional nucleic acid does not affect the induction of IL-2P by the signal transduction cascade X, Y, Z, and the expression of the HSV TK. The presence of the HSV TK and ganciclovir will lead to T-cell death due to the production of toxic compounds. No cell death will occur if the expressed RNA library contains functional sequences which affect the expression of HSV TK, for example by interrupting the signal transduction cascade by inhibiting one of its proteins. These cells can then be selected (see Fig. 8).

Figure 8: Same phenotypic selection scheme as in Figure 7 but in this case a functional nucleic acid is expressed from Pol III/vai/random/vai. (dashed arrows represent gene expression). The functional nucleic acid in this example binds to protein X and blocks the signalling from X to Y in the cascade. (straight arrows). Therefore, the induction of the IL-2 promotor is inhibited and no HSV TK expressed. The

T cell survives in the presence of ganciclovir and the gene coding for the functional nucleic acid can be isolated.

Figure 9: Vectors for the functional target identification.

A Scheme of important features of plasmid pF1; *CMV P*: promotor element "human cytomegalovirus immediate early promotor". Example of a constitutive promotor element that is active in a wide variety of cell types; *IL-2 P*: IL-2 promotor; *OriP*: Episomal replication. The Epstein Barr Virus (EBV) origin of replication and nuclear antigen (EBNA-1) allow the high copy episomal (extra chromosomal) replication and maintenance in primate and canine cell lines; *EBNA-1*: gene for the Epstein Barr virus nuclear antigen 1; *cDNA library*: The cDNA library in this example is derived from T cells. The library is used to select for the complementation of the inhibited signal transduction cascade. Overexpression of the factor that is inhibited by the functional nucleic acid bypasses the block in signalling in which case the IL-2 promotor is induced again in those cells which harbour the target gene of interest; *fNS-hsv thymidine kinase*: this construct allows the identification of the target gene product through the specificity of the nucleic acid-protein interaction. The gene of the functional nucleic acid (fNS), in this example an aptamer sequence, is cloned in the 5'-UTR of the herpes simplex virus thymidine kinase. Upon binding of the aptamer ligand (the functional nucleic acid) the expression of the reporter HSV-TK is inhibited presumably through blocking the translation; *neo*: Selectable marker and commonly used resistance gene (aminoglycosid phosphotransferase) to select for cells harbouring the transfected plasmids via detoxification of aminoglycosid antibiotics. **B** Scheme of important features of plasmid pF2. *CMV P*: see plasmid pF1; *Pol III P*: promotor element: RNA Polymerase III promotor; *vai fNS vai*: functional nucleic acid (fNS) with the adenoviral VAI RNA sequences that permit the export of the functional nucleic acid into the cytoplasm; *OriP*: See pF1; *hygromycin*: Resistance against hygromycin can be used to co-select for cells that harbor two plasmids (e.g. an additional plasmid with the neo resistance); *mIg*: Surface marker that allows the affinity

selection of transformed cells. For example, in this example the constant CH2 and CH3 domains of human IgG1 are expressed as a transmembrane version (m Ig) which is presented on the cell surface.

Figure 10: Scheme for the functional target identification. The functional nucleic acid identified in the phenotypic selection expressed from Pol III/vai/fNS/vai (dashed arrows represent gene expression) binds to protein X and blocks the signalling from X to Y (straight arrows) in the cascade after stimulation with phorbol-12-myristate-13-acetate (PMA) (see also Fig.8). As protein M (clone of a T cell cDNA library) which is expressed under the control of the promoter CMV P (see description of Fig. 9) has no effect on the blocked cascade, the induction of the IL-2 promoter (IL-2 P) is inhibited and hence the expression of the reporter constructs fNS hsv-thymidine kinase and mIg (see description of Fig. 9) does not occur. As the T cells do not express the mIg on their surface they can not be isolated using for example antibodies against mIg.

Figure 11: Scheme for the functional target identification. Same as Fig. 10, except that in this example the cognate target for the functional nucleic acid (fNS) protein X is expressed from the cDNA library (dashed arrows represent gene expression). The overexpression of protein X bypasses the block of the endogenous protein X by the fNS. Therefore, after stimulation with PMA, the signal transduction cascade X,Y,Z (straight arrows) is rescued and the mRNA is transcribed from IL-2 promoter reporter constructs. Expression of the surface mIg (see also Fig.9) allows the affinity isolation of the T cells. Simultaneously protein X binds to the fNS inserted in the 5'UTR of the fNS HSV TK construct. This results in the block of the translation of the HSV TK. The absence of the enzyme herpes simplex thymidine kinase enables the T cells to survive in the presence of ganciclovir and the positive cDNA clones can be isolated.

Figure 12. Scheme of the functional target identification. Although protein X is blocked by the functional nucleic acid (fNS) in this schematic example the protein N expressed from the cDNA library induces the Il-2 promotor reporter constructs by deregulating the endogenous signal transduction cascade by acting directly on protein Z (see also Figures 10, 11). This false positive complementation leads to the expression of the mIg which would allow the isolation of the cells with mIg specific antibodies. As protein N is not the cognate ligand for the functional nucleic acid it fails to bind to the fNS in the fNS-HSV TK mRNA construct (compare also Figures 10, 11). Therefore the herpes simplex thymidine kinase is expressed and the cells are killed in the presence of ganciclovir. This step eliminates false positive cDNA clones which act by deregulating the signal transduction.

The candidate RNAs or DNAs comprise functional nucleic acids i.e. a single stranded DNA (ssDNA) or RNA (ssRNA) or chemically modified forms thereof (ssNAMod), double stranded DNA (dsDNA) or RNA (dsRNA) or chemically modified forms thereof (dsNAMod) which are able to bind, modulate or catalytically modify an intracellular target thereby affecting its biological function. The functional nucleic acids can be identified, for example, by screening large combinatorial nucleic acid libraries (in vitro selection of ribozymes, aptazymes, aptamers etc.). Combinatorial nucleic acid libraries whether DNA or RNA usually contain a randomized sequence flanked by fixed regions which serve as primer annealing sites for enzymatic amplification via PCR or in the case of RNA via reverse transcription and PCR. The libraries can for example be synthesized as ssDNA on an automated DNA synthesizer using 3'phosphoramidite chemistry, whereby the randomized region is synthesized with 3'phosphoramidite solutions that contain mixtures of the four bases A,C,G,T (Hermes et al., Proc. Natl. Acad.Sci. U.S.A.87 (1990), 696-700). The ratios between the four

monomers can be varied depending on the application. For preparation of RNA the fixed regions contain a RNA polymerase promotor, e.g. T7 RNA polymerase promotor, which allows the transcription of the template DNA into RNA. If necessary the ssDNA can be made doublestranded using enzymatic protocols, e.g. PCR, which are well known to a person skilled in the art. For an overview see Abelson, *Methods in Enzymology* 267 (1996), pp 275-426, Academic Press, San Diego. Preferentially the library contains 8 to 200 randomized nucleotides (Tuerk and Gold, *Science* 249 (1990), 505-510; Bartel and Szostak, *Science* 261 (1993), 1411-1418) with complexities from 6×10^5 to 1×10^{16} which have been used in successful selection experiments. If desired, the functional nucleic acid can be covalently attached or non covalently bound to other molecules such as chemically reactive groups, lipids, peptides, proteins etc. which add additional properties to the functional nucleic acid. Functional nucleic acids do not include nucleic acids acting by nucleic acid interactions such as antisense mechanisms or natural ribozymes or modified and mutated forms thereof which combine antisense recognition with phosphodiester cleavage activity and are applied, for example, in gene therapeutic approaches. A functional nucleic acid can be part of a construct with additional sequences whether natural or non natural which add further functions, e.g. enhanced stability, localisation signals or enzymatic activities. Furthermore, upon binding its intracellular target the functional nucleic acid itself can be modulated in a second function whether intrinsic or added in a construct with additional sequences for example binding a third ligand or being catalytically active.

Aptamers are specific ligand-binding nucleic acid receptors (ssDNA, RNA, modDNA or modRNA) which can be isolated by screening the 'shape-space' of vast combinatorial libraries

of single stranded nucleic acids by in vitro selection. In vitro selection of aptamers can be achieved by contacting a nucleic acid library with a target, e.g. a protein, and partitioning the bound nucleic acids from the remainder of the nucleic acid mixture. Partitioning can be achieved by retaining the nucleic acid/protein complexes on nitrocellulose filters (Tuerk and Gold, *Science* 249 (1990), 505-510), immobilizing the target prior to incubation with the library on a solid support and removing non bound species through washes with an appropriate buffer (Nieuwieland et. al, *Biochemistry* 34 (1995), 5651-5659) or any other method which allows the separation of the target/nucleic acid complexes from unbound nucleic acid species. After isolation of the bound species DNA can be amplified using PCR or in the case of RNA reverse transcription, PCR and transcription. This cycle can be repeated several times to yield a library with increased affinity for the target. Subsequently individual aptamer sequences can be identified by cloning and sequencing the PCR products using standard methods.

Nucleic acid aptamers for far more than a hundred different targets have been described showing that aptamers can now routinely be obtained for almost every desired target (for reviews see e.g.: Osborne and Ellington, *Chem. Rev.* 97 (1997), 349-370; Famulok and Jenne, *Curr. Opin. Chem. Biol.* 2 (1998), 320-327).

The term ribozymes is used in the present invention for catalytically active nucleic acids that act on an intracellular target thereby modifying its functions. Not included in the present invention are naturally occurring ribozymes, such as hammerhead ribozymes, hairpin ribozymes or derivatives thereof which act by Watson Crick binding to nucleic acid substrates and phosphodiester cleavage and are used for example in gene therapeutic approaches (Bramlage, *Tibtech* 16 (1998), 435-438). Ribozymes with new catalytic properties can be isolated using in vitro selection experiments. For example ribozymes have been isolated which are able to catalize RNA alkylation, amide bond formation or

porphyrin metalation (Breaker, Chem. Rev. 97 (1997), 371-390).

Aptazymes can be viewed as chimeras between a catalytic RNA and an aptamer. These chimeras can be rationally designed or selected in vitro from certain combinatorial libraries (see:Robertson and Ellington, Nature Biotechnol. 17 (1999); 62-66). Usually, the aptameric domain of an aptazyme is used as a regulatory module for allosteric ribozyme activation or inhibition (Tang and Breaker, Chem. Biol. 4 (1997), 453-459; Tang and Breaker, RNA 3 (1997), 914-925; Tang and Breaker, Nucleic Acids Res. 26 (1998), 4214-4221; Araki et al., Nucleic Acids Res. 26 (1998), 3379-3384). Likewise, and consequently, if an aptazyme is selected to be affected by a regulatory molecule it contains a binding domain for this molecule and thus can also act like an aptamer.

Intramers are functional nucleic acids (aptamers, aptazymes etc.) that are delivered exogenously or endogenously into procaryotic or eucaryotic cells and bind, modulate or enzymatically modify an intracellular target.

Modulation of an intracellular target can be, for example, the inhibition or stabilization of a protein/protein interaction or protein-domain/protein interaction, or protein/nucleic acid interaction, or protein/small molecule interactions etc. Modulation can also include activation or inhibition of enzymes via different mechanisms such as sequestering the substrate(s), binding to the active site, induction of conformational changes, allosteric regulation etc. Modulation of an intracellular target can occur both by non-covalent interactions or covalent modifications such as biotinylation (Wecker et al., RNA 2 (1996), 982-94). Further methods developed for example for antibody based approaches could be adapted to the functional nucleic acid system in which case the aptamer could be conjugated to a molecule that is able to chemically inactivate the bound target. For example, the intracellular functional nucleic acids are tagged via a short annealing sequence which is covalently attached to the dye malachit green (MG). These short

oligonucleotides could be applied exogenously. MG absorbs light at a visible wavelength which is not significantly absorbed by cells. After irradiation with a laser the MG becomes activated and generates short lived hydroxyl radicals which inactivate proteins most likely by modifying amino acid side chains ["Chromophore assisted laser inactivation (CALI)" see: Liao et al., Proc. Natl. Acad. Sci. 91 (1994), 2659-63]]. The inactivation process occurs only at a very short distance from the dye moiety. Thus, the targeted protein or subunit can generally be damaged without affecting its neighbours. This has two advantages: First, the aptamer would not necessarily have to bind to the active site of the protein but could interact with a neighbouring epitope that might be better suited for binding to a nucleic acid. Even though the bound epitope is distant from the active site the protein will still be specifically inactivated. Second, as the protein is irreversibly inactivated and the aptamer can be constitutively expressed, several pulses of irradiation could inactivate high abundance proteins that are initially present at higher copy numbers than the specific aptamer.

Intracellular targets include small molecules, biological cofactors, natural polymers such as proteins, protein domains, peptides, and nucleic acids, glycoproteins, hormones, receptors, protein complexes, protein/nucleic acid complexes, toxins, viruses, and other compounds that are naturally found inside cells.

Phenotype can be for example the induction or repression of the transcription of a certain gene, processes linked to signal transduction cascades such as adhesion in response to certain stimuli, differentiation of cells, apoptosis etc.

Phenotypic selection is a process in which nucleic acid libraries are introduced into cells by various methods known to a person skilled in the art (electroporation, lipofection, expression with viral vector systems, expression from plasmids containing RNA polymerase promoters

etc.). Those cells containing a functional nucleic acid acting on a target which is involved in a certain phenotype are separated on the basis of their altered phenotype from the bulk cellular material which do not show an altered phenotype. Cells used in the phenotypic selection include procaryotes, yeast, insect- or plant-cells and preferentially mammalian cells (e.g. CHO, HeLa, COS, MDCK, 293, WI38 and Jurkat E6 cells). If a clear separation on the basis of the altered phenotype is not possible or difficult, reporter constructs can be applied which allow a more definite or better discrimination. In a positive phenotypic selection experiment, for example the induction of a cellular promoter or enhancer/promotor construct, a reporter gene which allows the identification of the altered phenotype (here the induction of the promoter) is inserted under the control of the inducible promoter in the reporter construct. Reporter genes used in this type of selection are for example resistance genes like aminoglycoside phosphotransferase (Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327-341) which inactivates aminoglycoside antibiotics (neomycin, G418) making cells resistant to this drug. Consequently, if the inducible promoter is turned on through the action of a functional nucleic acid (fNS), the cells harbouring the gene for the functional nucleic acid will survive the presence of the drug and the genes for the fNS can be identified. For example mammalian promoters which are dependent on activation via the transcription factor NF κ B could be activated by inhibiting the I κ B proteins which normally prevent the import of NF κ B into the nucleus (Rolfe et al., J.Mol.Med. 75 (1997), 5-17; Fuchs et al., Oncogene 17 (1998), 1483-90). Other examples of promoters which are regulated by an inhibitory mechanism are bacterial transcription units, for example the E.coli lac operon

regulated by the lac repressor (Gilbert and Muller-Hill, Proc.Nat.Acad.Sci. 56 (1966), 1891-1898).

Negative phenotypic selection can occur for example by inhibiting the induction of a promotor. In this case the reporter gene could be a toxin (e.g. Herpes simplex virus thymidine kinase (Fife et al., Gene Ther. 5 (1998), 614-620) or diphtheria toxin (Massuda et al., Proc.Natl.Acad.Sci. U.S.A. 94 (1997), 14701-14706)), which promotes the death of cells where no functional nucleic acid inhibits the cellular signal transduction pathway which induces the promotor. Inducible genes include bacterial, viral and eukaryotic transcriptional units, e.g. in the arabinose operon (Wilcox et al., J.Biol.Chem 249 (1974), 2946-2952), the vaccinia virus early and late genes (Moss, Annu. Rev. Biochem. 59 (1990), 661-688) or promoters of immunoglobulin genes (Staudt et al., Ann. Rev. Immunol. 10 (1991), 373-398). The promoters used in the phenotypic selections may be naturally occurring promotor/enhancer elements or any combinations or modifications thereof.

Reporter constructs can include positive or negative phenotypic selections. For example, negative phenotypic selection can occur via the expression of a toxin (such as the diphtheria toxin) under the control of an inducible cellular promotor such as the interleukin-2 promotor in T-lymphocytes. These gene constructs can be introduced into cells e.g. via plasmids and the expression of the toxin-gene will be driven by the cell's natural promotor-inducing machinery. Only in those cells in which a functional nucleic acid inhibits the induction of the toxin-promotor the toxin will not be expressed and the cells survive and can be selected.

The vectors (vector elements) and protocols for cloning, transfecting, transient gene expression and obtaining stable transfected cell lines are well known and described in the literature (Maniatis et al. (1989) Molecular Cloning: A

Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbour, New York; Spector et al., Cells: A Laboratory Manual (1998), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Vectors for the expression of the nucleic acid libraries can be any commonly used constructs to introduce foreign genes into cells, for example linear PCR fragments, plasmids, DNA viruses like M13 or vaccinia, RNA viruses like retroviruses or the Semliki Forest virus. The genes for the nucleic acid library are placed under the control of suitable promoters, preferentially those, which allow high level transcription of the nucleic acid genes.

Examples are but are not limited to polymerase III dependent promoters such as yeast RNase P promotor (Good and Engelke, Gene 151 (1994), 209-214), mammalian U6 or tRNA^{met} promoters (Bertrand et al., RNA 3 (1997), 75-88), retroviral long terminal repeats (Rossi, Tibtech 13 (1995), 301-306), T7 RNA polymerase promoters as described in Example 1 or the subgenomic promotor for the Semliki Forest virus replicase (Tubulekas et al., Gene 190 (1997), 191-195).

Vectors used as reporter constructs can be plasmids (e.g. pUC18, pYES2, pCDM8) or viral genomes such as adenovirus, AAV or retroviral vectors (Moloney murine leukemia virus (MoMuLV), gibbon ape leukemia virus (GaLV), etc. Using stably transfected reporter cell lines is optional but provides a reliable system to select for cells which survive the negativ selection in the presence of a toxin.

Target validation is the identification of a given cellular target as a key molecule responsible for a given phenotype. It involves the intracellular application of a nucleic acid modulator which affects the target to investigate its role in the complex network of cellular components. The preferential goal is to investigate whether or not the

target is responsible for a certain cellular status or disease state and thus is a good drug target.

Target identification is the linkage of a cellular component to a given phenotype regardless whether its biological function is known or not. Target identification can occur by means of a functional nucleic acid modulator which has been identified by phenotypic selection; the target affected by such a modulator is likely to represent the or a key molecule responsible for a certain phenotype. The target can be identified through its interaction with the functional nucleic acid. For example the yeast three-hybrid system, a modification of the two-hybrid system, is used to detect RNA-protein interactions. The three-hybrid system utilizes an RNA hybrid as an intermediate between two hybrid proteins. The interaction of the three components serves to reconstitute transcriptional activation of reporter genes in a mechanism similar to the two-hybrid system (SenGupta et al., Proc. Natl. Acad. Sci. 93 (1996), 8496-8501). The functional nucleic acid cloned in the RNA hybrid will interact with its target protein expressed as part of a protein-hybrid library of cellular proteins. Therefore the reporter genes will be expressed in those yeast cells which harbour both, the functional nucleic acid and its target, and the plasmids with the target genes can be isolated.

Another approach is to identify the target via complementation cloning as outlined in Example 2. This technique has been applied successfully to isolate proteins which functionally complement cell lines bearing mutated endogenous genes, for example complementation cloning of NEMO a component of the I κ B kinase complex (Yamaoka et al., Cell 93 (1998), 1231-1240).

Highly specific nucleic acid modulators of cellular target molecules can be routinely obtained for example by application of the technologies SELEX, aptazymes, two- and three-hybrid technologies, the phenotypic selection method described herein etc. The resulting nucleic acid modulators can be brought into cells exo- or endogenously by methods such as electroporation, lipofection, expression with viral vector systems, expression from plasmids containing RNA polymerase promoters etc. Functional nucleic acids can act on their target molecule by different mechanisms; they can either bind their target molecule in an antibody-like fashion thereby modulating its biological function. For example, chemically modified nuclease resistant aptamers have been selected for binding to extracellular growth factors such as basic fibroblast growth factor (bFGF; see: [Jellinek et al., Proc. Natl. Acad. Sci. USA 90 (1993), 11227-11231; Jellinek et al., Biochemistry 34 (1995), 11363-11372.]), human keratinocyte growth factor (hKGF; see: [Pagratis et al., Nature Biotech. 15 (1997), 68-73]), platelet derived growth factor (PDGF; see: [Green et al., Biochemistry 35 (1996), 14413-14424]), or vascular endothelial growth factor (vEGF; see: [Green et al., Chem. Biol. 2 (1995), 683-695; Jellinek et al. Biochemistry 33 (1994), 10450-10456; Ruckman et al., J. Biol. Chem. 273 (1998), 20556-20567]). These aptamers blocked the binding of their extracellular target to its natural protein receptor thus modulating the growth factor's biological function.

While it is state of the art to modulate the biological function of an extracellular protein, a central aspect of the present invention is the exploitation of the huge potential of high affinity and highly specific nucleic acid reagents to modulate a given target for intracellular applications. That this can indeed be done was shown representatively in the system exemplified in Example 1:

An approach is described which is based on integrin cytoplasmic domain recognition by synthetic ligand-binding RNA-aptamers isolated from a combinatorial RNA library. A vaccinia virus-based RNA expression system enabled high-

level cytoplasmic expression of RNA aptamers (intramers) directed against the intracellular domain of the $\beta 2$ integrin LFA-1, a transmembrane protein which mediates cell adhesion to intercellular adhesion molecule-1 (ICAM-1). In two different cell types, cytoplasmic expression of integrin-binding intramers reduced inducible cell adhesion to ICAM-1. The aptamers specifically target, and thereby define a functional cytoplasmic subdomain important for the regulation of cell adhesion in leukocytes.

Integrins are versatile heterodimeric transmembrane proteins which mediate adhesive interactions with extracellular matrix and cell specific counter-receptors. They are implicated in diverse biological processes including apoptosis, cell-cycle regulation, cell migration, bloodclotting, memory and leukocyte function (Hynes Cell 69 (1992), 11-25). The cytoplasmic domains of integrins play important roles in regulating cell adhesion (Diamond and Springer Curr. Biol. 4 (1994), 506-517; Dedhar and Hannigan Curr. Opin. Cell Biol. 8 (1996), 657-669), probably by linking signaling events inside the cell to the extracellular domains, but the precise mechanisms are unclear. The $\beta 2$ integrin LFA-1 ($\alpha L\beta 2$, CD11a/CD18) mediates adhesion of leukocytes in immune and inflammatory responses by binding to cellular ligands (Lub et al., Immunol. Today 16 (1995), 479-483), the intercellular adhesion molecules ICAM-1, -2, or -3. A large body of data implicates the cytoplasmic domain of the $\beta 2$ -chain in the regulation of LFA-1 mediated adhesiveness, presumably through its interactions with intracellular proteins and/or the membrane-proximal cytoskeleton (Stewart et al. J. Cell Biol. 140 (1998), 699-707), and some candidate cytoplasmic ligands have recently been identified (Hibbs et al., J. Exp. Med. 174 (1991), 1227-1238; Pavalko and LaRoche, J. Immunol. 151 (1993), 3795-3807; Sharma et al., J. Immunol. 154 (1995), 3461-3470; Kolanus et al., Cell 86 (1996), 233-242). To gain further insights into these mechanisms a system was devised allowing the specific inhibition of the LFA-1 mediated inside-out signal transduction in vivo. The objectives were the

02849 02849 02849

selection of aptamers which bind to the cytoplasmic domain of CD18, the development and application of a system allowing high-level expression of aptamers within the cytoplasm of leukocytes, and investigations into their biological effects in the context of the living cell.

Briefly, as detailed in Example 1 below, the inventors were able to

a) isolate RNA aptamers which bind to an immobilized peptide corresponding to the cytoplasmic domain of the integral transmembrane protein LFA-1 (CD18, β 2-integrin)

b) show that the aptamers recognize their target specifically even when cloned into a different sequence context in an RNA expression cassette. This cassette was derived from a T7 RNA polymerase dependent RNA expression system (Fuerst and Moss, J. Mol. Biol. 206 (1989), 333-348) and contained a 5'-stem-loop structure which has previously been shown to stabilize RNA molecules in the cytoplasm by preventing their degradation. Furthermore, the 3'-end contained the T ϕ -terminator hairpin loop motif as a terminator for T7 RNA polymerase dependent transcription. Another example for a cytoplasmic system, which might be suitable for RNA expression, is based on the RNA dependent RNA Replicase of viral vectors like Semliki Forest virus (SFV) (Tubulekas et al., Gene 190 (1997), 191-195). Most importantly, it was shown that the aptamer transcripts resulting from the T7 RNA polymerase-expression cassette template were correctly terminated and fully retained their binding activity to CD18cyt. Incorrectly terminated aptamer sequences which contained additional sequence information from the viral genome for binding to CD18cyt were also tested. It was found that this undesired additional sequence information resulted in a severely reduced binding affinity of these aptamer variants to CD18cyt in vitro probably due to interfering with correct aptamer folding. This result establishes two very important and surprising points: i) it shows that it is possible to express the aptamer in the context of additional sequence information such as

stabilizing sequence motifs or RNA localization signals etc. which is crucial for broad application of endogenous aptamer-expression ii) it shows that it is possible to engineer small, compact aptamers selected in vitro in a modular fashion providing the aptamers with additional properties ensuring proper and efficient intracellular applicability without affecting aptamer-binding to the target.

c) show that the selected aptamers specifically recognize endogenous LFA-1 in crude Jurkat E6 cell lysate. They do so also in the context of the RNA expression cassette.

d) develop a vaccinia virus-based RNA expression system which enabled high-level cytoplasmic expression of RNA aptamers directed against the cytoplasmic domain of the integrin LFA-1.

e) alter the phenotype of two different cell types, Jurkat E6 and peripheral blood mononuclear cells (PBMC) by cytoplasmic expression of aptamers binding the cytoplasmic domain of LFA-1 in these cells; in this case cytoplasmic aptamer expression specifically reduced inducible cell adhesion to intercellular adhesion molecule 1 (ICAM-1). This result is unexpected and represents one of the central aspects of this invention for several reasons: i) for the first time the intracellular target is a protein or protein domain without any known natural affinity to nucleic acids. The novel and unexpected aspect of this finding is that the in vitro selected aptamer specifically finds the target it has been selected for in an intracellular compartment which is very different from other compartments because it contains a huge number of professional RNA-binding proteins (such as helicases, ribosomal proteins, hnRNP proteins etc.) which exist in high copy. numbers of several million per cell. These professional RNA-binding proteins can bind with very high affinity (K_d 10^{-9} - 10^{-8} M) to special RNA motifs but can also interact with RNAs in a more sequence independent manner with still relatively high affinities [e.g. the hnRNP

proteins A1 and C1; K_d 10^{-7} - 10^{-6} M; see: Nagai and Mattaj (Eds.), RNA-Protein Interactions (1994), Oxford University Press, 138-140]. Despite the expectation that these professional RNA-binding proteins compete with the cognate aptamer-target for binding to the aptamer it was shown here that the aptamer can find its target inside the cell and change the cell's phenotype. ii) although the in vitro affinity of the aptamer to its cognate CD18cyt protein domain was relatively low (K_d 10^{-6} - 5×10^{-7} M) it recognizes its target with unexpected specificity. This shows that this system is not restricted to targets which are recognized by an aptamer with affinities that are significantly higher than the unspecific interactions between a professional RNA-binding protein and a nucleic acid. This opens up the exciting possibility to generally apply aptamers even if they bind their targets with relatively low affinity. iii) it has been shown for the first time that functional nucleic acid ligands can act in cellular sub-compartments in which nucleic acids normally do not appear. In this particular case the aptamer finds its target even though it is anchored to the negatively charged plasma membrane including its associated compact cytoskeletal protein cortex. It could not be expected in advance that the target would be accessible by the aptamer at these sites.

f) map the binding site of the aptamers to the cytoplasmic domain of LFA-1 (CD18cyt) by employing synthetic peptides comprising overlapping regions of the cytoplasmic domain of LFA-1. Thereby, the aptamer binding site on CD18cyt was defined as functionally important for stimulated cell adhesion of LFA-1 to ICAM-1.

Most of our current knowledge regarding factors and mechanisms that control integrin activation so far stems from mutational analyses of integrin domains. This study shows that it is now feasible to address this issue by retaining the genetic integrity of the wild-type CD18 cytoplasmic domain. For the first time it was shown that the intracellular expression of CD18cyt-specific aptamers can

affect the functional state of endogenous LFA-1 molecules. Furthermore, evidence is provided that these "intramers" target a specific subdomain of the $\beta 2$ cytoplasmic tail implicating the importance of this region for the proper function of the endogenous LFA-1 receptor with respect to its adhesion to ICAM-1.

Current state of the art technologies are able to produce nucleic acid ligands which bind almost any given target with high affinity and specificity. Extension to automation of the selection procedures may even speed up the generation of such compounds. Although the process of in vitro selection of nucleic acid aptamers is currently done largely manually, the first fully automated selection protocol has been reported and it is suggested that in the future it would potentially allow the isolation of hundreds of nucleic acid ligands within a few days making it much more efficient than screening small molecule compound libraries for possible effectors (see Cox et al., Biotechnol. Prog. 14 (1998), 845-50).

The method described above employs these specific nucleic acid modulators to determine the role of an intracellular component in the complex intracellular regulatory network or to find out whether it is the causal reason for a given phenotype. Furthermore, it is a powerful system which enables the validation of potential drug targets.

The intracellular application of functional nucleic acid ligands can easily be achieved. Depending on the suitability and the questions asked, the functional nucleic acids can be delivered either endogenously or exogenously. In the latter case, chemically modified forms that prevent the degradation of the functional nucleic acid are preferentially applied. Stabilizing modifications include, for example, RNA molecules in which the 2'-hydroxy function of pyrimidine bases is substituted by a 2'-amino or 2'-fluoro residues. Endogenous transcription expression systems such as the one described in Example 1 allow the efficient intracellular expression of functional nucleic acids. Depending on the

application and site of action, appropriate vectors and promoters known to the expert are chosen. The functional nucleic acids can be engineered with additional nucleic acid domains to ensure proper function and localization. Besides the demonstrated suitability of the method for cell culture systems it should be possible to extend it to transgenic organisms such as plants or animals that have the genes for the nucleic acid modulators integrated into their genomes. A commonly used method for producing transgenic animals is the introduction of foreign DNA (e.g. plasmids) into fertilized oocytes via microinjection. The vectors often contain reporter genes, for example the lacZ gene, to allow the identification of the transgenic organisms (see for example Brinster et al., Nature 306 (1983), 332-336; Brinster et al., Cell 27 (1981), 223-231). The oocytes are brought into the uterus of pseudopregnant hosts. The animals developing from the transfected oocytes are analyzed for the presence of the gene using methods like Southern Blot analysis. The primary animals will be heterozygous for the foreign gene. To obtain homozygous individuals two heterozygous animals are bred and the second generation analyzed for homozygous offspring. Other vectors for gene transfer include retroviral vectors for mammalian cells or Ti plasmids for plants. In general this technique should be useful to introduce genes of functional nucleic acids into the host's genome. This should allow to investigate in vivo effects induced by the modulated target in the otherwise unaltered cellular status in the context of a whole multicellular organism. The ease by which the nucleic acid modulators can be delivered and engineered makes this system superior to similar protein based methods currently available. It is advantageous, for example, that RNA processing, efficient translation, chaperone-assisted folding, posttranslational modification, subunit assembly etc. which are crucial for proper protein function are circumvented with the more simple nucleic acid based system. The nucleic acid-based method thus could significantly contribute to the field of "functional genomics".

In a second aspect the present invention relates to methods for the expression of randomized nucleic acid libraries inside cells for the identification of functional intramers which alter the phenotype of the cell in which they are expressed. That this is feasible is a direct consequence of the effectiveness of the system as described above. This target identification approach is useful for directly establishing a cellular component as important for or involved in a particular phenotype such as a certain status associated with a disease. Based on current knowledge it can be estimated that a nucleic acid library expressed inside cells contains individual functional nucleic acids which are able to specifically recognize a cellular component. Based on the findings described in the first part of this invention it can also be expected that this specific recognition is maintained or is generally possible within the cellular compartment. For example, the library should contain molecules which are able to bind or modify activators or inhibitors of signal transduction pathways thereby modulating phenotypes associated with the cascade. In such a case the cell containing the functional nucleic acid can be isolated by phenotypic selection (see definition above). If a clear separation on the basis of the altered phenotype is not possible or difficult, reporter constructs can be applied which allow a more definite or better discrimination. This can be done without prior knowledge regarding the nature of the target or its biological function. Thus, the first step of this process is the phenotypic selection. The nucleic acid library is introduced into the cell for example via expression vectors. Phenotypic selection can be achieved preferentially via reporter constructs either by positive or negative selection. Positive phenotypic selection could occur, for example, by expression of an appropriate cell surface marker and subsequent affinity isolation of the cell with a surface marker-specific antibody. Negative phenotypic selection can occur via the expression of a toxin (such as the diphtheria toxin or herpes simplex virus thymidine kinase) under the control of an inducible cellular promoter. These gene constructs can be introduced into cells e.g. via plasmids

and the expression of the toxin-gene will be driven by the cell's natural promotor-inducing machinery. Only in those cells in which a functional nucleic acid inhibits the induction of the toxin-promotor the toxin will not be expressed and the cells survive and can be selected. After enrichment of the selected phenotype the functional nucleic acid modulators are identified by sequencing and tested individually for functionality. Once the functional nucleic acid has been identified and established it can in turn be applied to identify its intracellular interactor. This can be achieved, for example, by standard methods for investigating protein/nucleic acid interactions such as screening proteins expressed from cDNA-libraries according to the yeast three hybrid system to detect RNA/protein interactions in vivo (see: SenGupta et al., Proc. Natl. Acad. Sci. 93 (1996), 8496-8501). Preferentially, the identification occurs via complementation in homologous cells. By expression of cDNA libraries in homologous cell systems clones can be isolated which complement the signal transduction cascade modulated by the functional nucleic acid. At the same time, reporter systems can be constructed in cells in which the aptamer/target interaction is confirmed by systems such as the three hybrid system (see: SenGupta et al., Proc. Natl. Acad. Sci. 93 (1996), 8496-8501) or the approach described in Example 2 (see below) for the positive selection incorporating negative selection proofreading. This provides a dual read-out for the reliable identification of those proteins which play a key role for a particular phenotype in a wild-type context.

In addition, the phenotypic selection system also identifies nucleic acid inhibitors which could simply be applied for target validation (see first part of this invention). This method, thus, significantly contributes to the field of functional genomics/proteomics because it identifies targets directly in an unaltered cellular status by their function rather than their genes or their presence in a cell. Therefore this method of the present invention is superior over the current state of the art.

The identified functional nucleic acids can serve as drug leads or can be used as therapeutics themselves, particularly in gene therapeutic approaches.

Thus, the present invention also relates to a pharmaceutical composition comprising a functional nucleic acid identified by the methods of the present invention which is capable of binding to and modifying the function of the functional intracellular target, optionally in combination with a suitable pharmaceutical carrier.

Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The route of administration, of course, depends on the nature of the disease and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind of the disease, general health and other drugs being administered concurrently.

Preferably, the functional nucleic acid (e.g. intramer) is administered directly to the target site, e.g., by biolistic delivery, as a colloidal dispersion system or by catheter to a site in artery. The colloidal dispersion systems, which can be used for delivery of the pharmaceutical composition of the invention include macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, (mixed) micelles, liposomes and lipoplexes. The preferred colloidal system is a liposome.

The composition of the liposome is usually a combination of phospholipids and steroids, especially cholesterol. The skilled person is in a position to select such liposomes which are suitable for the delivery of the desired nucleic acid molecule. Organ-specific or cell-specific liposomes can be used in order to achieve delivery only to the desired site. The targeting of liposomes can be carried out by the person skilled in the art by applying commonly known methods. This targeting includes passive targeting (utilizing the natural tendency of the liposomes to distribute to cells of the RES in organs which contain sinusoidal capillaries) or active targeting (for example by coupling the liposome to a specific ligand, e.g., an antibody, a receptor, sugar, glycolipid, protein etc., by well known methods). In the present invention monoclonal antibodies are preferably used to target liposomes to specific sites via specific cell-surface ligands.

In a still more preferred embodiment the functional nucleic acid molecule (e.g. intramer) is integrated into a recombinant vector, preferably an expression vector. In order to achieve expression only in the target organ, the functional nucleic acid molecule encoding DNA is operably linked to a tissue specific promotor and used for gene therapy. For example, it can be ligated to the CD4 gene promotor (Zhao-Emonet et al., Biochim. Biophys. Acta. 1442 (1998), 109-119), secretory leukoprotease inhibitor gene promotor (Robertson et al., Cancer Gene Ther. 5 (1998), 331-336) or carcinoembryonic antigen promotor (Fichera et al., Dis. Colon. Rectum. 41 (1998), 747-754). Other tissue specific promoters are well known to those skilled in the art.

Preferred recombinant vectors are viral vectors, e.g. adenovirus, herpes virus, vaccinia, or, more preferably, an RNA virus such as a retrovirus. Even more preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of such retroviral vectors which can be used in the present invention are: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcome virus (HaMuSV), murine

mammary tumor virus (MuMTV) and Rous sarcoma virus (RSV). Most preferably, a non-human primate retroviral vector is employed, such as the gibbon ape leukemia virus (GaLV), providing a broader host range compared to murine vectors. Since recombinant retroviruses are defective, assistance is required in order to produce infectious particles. Such assistance can be provided, e.g. by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. Suitable helper cell lines are well known to those skilled in the art. Said vectors can additionally contain a gene encoding a selectable marker so that the transduced cells can be identified. Moreover, the retroviral vectors can be modified in such a way that they become target specific. This can be achieved, e.g., by inserting a polynucleotide encoding a sugar, a glycolipid, or a protein, preferably an antibody. Those skilled in the art know additional methods for generating target specific vectors. Further suitable vectors and methods for in vitro- or in vivo-gene therapy are described in the literature and are known to the persons skilled in the art; see, e.g., WO 94/29469 or WO 97/00957.

The present invention also relates to a pharmaceutical composition containing a compound which has the same or an even increased effect compared to the original functional nucleic acid (intramer) identified by the methods of the present invention. A strategy for increasing the stability and improving the binding specificities of the functional nucleic acids identified by the methods herein has been described as post-SELEX modification and deconvolution of post-SELEX modified aptamers (Eaton, Cur. Opin. Chem. Biol. 1 (1997), 10-16). After identification of the functional nucleic acid a secondary nucleic acid library is derived from the parental sequence by combinatorial chemical synthesis and random incorporation of modified nucleosides. For example, the purine residues could be synthesized with mixtures of 2'-OH and 2'-O-methyl substituted nucleoside phosphoramidites. The ratios can be varied and will depend on the desired application. Modified nucleosides which can

be included in the chemical synthesis are well known to a person skilled in the art and include, for example, modifications at the 5-position of pyrimidines, 8-position of purines or the 2'-position of all nucleotides. Modifications include but are not limited to vinyl-, prenyl-, fucose-, biotin- or arginine-groups, adding additional chemical properties to the nucleic acid. In a second SELEX-experiment the modified secondary library is contacted with the ligand and those clones identified which exhibit the same or increased binding properties compared to the parental sequence. The identification of the modified positions in the winning oligonucleotides can be achieved, for example, via alkaline hydrolysis interference experiments because 2'-O-methyl substitutions protect the adjacent phosphodiesterbond from alkaline hydrolysis. Using this method secondary aptamers against vascular endothelial growth factor (VEGF) have been identified which bind VEGF with a 17-fold increased affinity probably due to additional hydrophobic contacts mediated via the methyl substitutions and 8 fold increased stability against degradation ($t_{1/2}$ = 171h) in undiluted rat urin (Green et. al., Cur. Biol. 2 (1995), 683-695). Thus increased efficiency (e.g. better affinities or better cellular uptake) can be added to the nucleic acids by chemical substitutions and the stabilized forms could be useful for the exogenous delivery of the functional nucleic acids as pharmaceutical formulations, encompassing the need to introduce the genes for the functional nucleic acids into the target cells.

Moreover the functional nucleic acids could be used as functional leads to screen small molecule libraries for new drugs. If the functional nucleic acid acts for example as a inhibitor of an enzyme through binding the active site, this interaction can be used to screen for small molecules which bind at the same site and act as a mimetic of the functional nucleic acid. Further binding to the same site suggests that the molecule can act in the same fashion as the functional nucleic acid, namely inhibiting the enzymes function. Those small molecule mimetics could be identified, for example, by

variations of the three hybrid system and reverse two hybrid system. As described in the target identification process two hybrid proteins and one hybrid RNA form a complex in the three hybrid approach and activate gene transcription in yeast. In the reverse two hybrid system reported by Leanna and Hannik, Nucleic Acid Res. 24 (1996), 3341-3347, the reporter gene CHY2 is transcribed through the activation of transcription mediated by the interaction of two hybrid proteins. This results in cell death of yeast cells in the presence of cycloheximide. In a three hybrid system with similar reporter constructs designed for the interaction of the functional nucleic acid and in this example the target enzyme only the inhibition of the assembly of the three hybrid components will promote cell survival. Consequently this assay can be used to screen small molecule libraries for mimetics which compete with the functional nucleic acid for binding to the target enzyme, therefor preventing the formation of the transcription activating complex. These compounds can further be assayed for their ability to functionally inhibit the enzyme and can serve as drugs or drug leads.

The functional nucleic acids of the invention or the compounds described above are particularly useful for the preparation of a medicament for gene therapy. They could be used besides other examples for the treatment of diseases which have been proposed to be targets for the application of intracellular antibodies, for example HIV infections (Chen et al., Human Gene Ther. 5 (1994), 595-601) or leukemias, breast and ovarian carcinomas (Richardson and Marasco, Tibtech 13 (1995), 306-310)

Finally, the present invention also relates to a kit for the diagnosis of diseases which are associated with a particular intracellular target as discussed above. Such a kit can be used in order to determine whether this target is present, has an abnormal concentration, etc. In a preferred embodiment, said kit contains the functional nucleic acid which is capable of binding to the target or, alternatively,

an antibody having the same or a similar target specificity, thus allowing diagnosis by ELISA. The antibody is bound to a solid support, for example, a polystyrene microtiter dish or nitrocellulose paper, using techniques known in the art. Alternatively, said kits are based on a RIA and contain said antibody marked with a radioactive isotope. In a preferred embodiment of the kit of the invention the antibody is labelled with enzymes, fluorescent compounds, luminescent compounds, ferromagnetic probes or radioactive compounds.

Example 1: Cytoplasmic RNA-modulators of an inside-out signal transduction cascade

(a) In vitro selection of CD18-binding RNA aptamers.

An RNA library with a complexity of $5 \cdot 10^{14}$ different molecules was synthesised by *in vitro* T7-transcription from the PCR-amplified synthetic DNA pool MF76.1: 5'-TCTAATACGACTCACTATAGGGCGCTA AGTCCTCGCTCA-N40-ACGCGCGACTCGGATCCT-3'; primer MF39.1: 5'-TCTAATACGACTCACTATAGGGCGCTAA GTCCTCGCTCA-3' (italic: T7-promotor, bold: BamHI restriction site); primer Mic20.1: 5'-GTAGGATCCGAGTCGCGCGT -3' (bold: BamHI restriction site) as described previously (Klug et al., Proc. Natl. Acad. Sci. USA 94 (1997), 6676-6681). CNBr-activated sepharose was derivatized with synthetic peptides (CD18cyt, MF2G), or blocked with Tris-HCl (pH 8.0) alone according to the manufacturers protocol (Pharmacia Biotech AB, Uppsala, Sweden). For the pre-selection, 50-75 μ l of either Tris-blocked- or MF2G-derivatized Sepharose was incubated with 2-3 nmol α - 32 P-labeled RNA in 150 μ l of binding buffer (buffer B: 4.3 mM K_2HPO_4 , 1.4 mM NaH_2PO_4 , 150 mM $NaCl$, 1.0 mM $MgCl_2$, 0.1 μ M $CaCl_2$) for 1 h at 23° C. The slurry was transferred into a plastic column and eluted in small fractions (75 μ l) with buffer B. For the actual selection the initial four fractions were precipitated and incubated with 25-50 μ l CD18cyt-Sepharose (4.5 mg/ml gel) in 100 μ l buffer B for 1 h at 23° C. Removal of non-binding RNAs was achieved by

washing with 200 Sepharose-volumes of binding buffer (5-10 ml). Bound RNAs were eluted by washing with buffer B adjusted to 6 M guanidinium-HCl. Eluted fractions were quantified by Cherenkov counting, ethanol-precipitated in the presence of 10-20 µg glycogen, and reverse transcribed as described previously (Famulok, J. Am. Chem. Soc. 116 (1994), 1698-1706); cDNA was PCR-amplified, followed by in vitro transcription. For the first cycle 29 nmol RNA and 120 µl CD18cyt-Sepharose in a total volume of 475 µl were used. The RNA library was subjected to 11 cycles of *in vitro* selection by binding to a 46-mer peptide corresponding to the complete cytoplasmic domain of CD18 (CD18cyt) immobilised on a sepharose matrix (Figure 1A). After cycle 11, binding sequences were PCR-amplified with primers Mic20.1 and SK40.4 (5'-TCTAATACGACTCACTATAGGGCTGCAGAGTCCTCGCTCA-3'; *italic*: T7-promotor, **bold**: PstI restriction site) to introduce a Pst I restriction site, cloned, and sequenced as described previously (Famulok, J. Am. Chem. Soc. 116 (1994), 1698-1706). From the enriched pool, 80 aptamers were cloned and sequenced. Based on sequence motifs these aptamers can be subdivided into either four classes or orphan sequences without any obvious relationship to each other.

(b) Construction of the cytoplasmatic aptamer-expression-cassette

Four sequences were chosen for the cytoplasmic expression studies. Three of them, D20, D28, and D31 are CD18-binders, the fourth sequence, D42, is a non-binding negative control sequence of the same length and with primer binding sites identical to the specific aptamers (Figure 1B). First, a T7-RNA expression cassette (TR) vector was constructed where the T7 promoter is located upstream of aptamer-coding DNA inserted between 5'- and 3'-stem-loop structures which serve as RNA-stabilising motifs and are required for correct termination of the T7-transcripts (Fuerst and Moss, J. Mol. Biol. 206 (1989), 333-348) (Figure 2A). The sequence of the T7-RNA expression cassette, TR, shown here without inserted aptamer (5'-GTTAAC-GCATGCTAATACGACTCACTATAG GGAGACCACAACGGT TTCCCGGGCGCAAGTTACTAGT-TGGCCA-AGATCT-TAATTAATAGCATAACCCCTTGG

GGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGTCGAC-GCGGCCGC-3'; **bold:**
restriction sites in the order in which they appear HpaI,
SphI, XmaI, SpeI, BalI, BglII, PacI, SalI, NotI; *italic:* T7-
promotor; underlined: stabilizing 5'stem-loop and 3'
Terminator T ϕ for correct termination of the TR-aptamers) was
inserted into the vector pTkg (Romeo and Seed, Cell 64
(1991), 1037-1046; Falkner and Moss, Virol. 62 (1988), 1849-
1854) via HpaI and NotI restriction sites resulting in the
transfer TR vector. Aptamer-encoding sequences were inserted
via the XmaI and PacI restriction sites (TR-aptamer; for an
overview see Figure 2A). The resulting transcripts are
called TR-aptamers. As negative controls, a TR vector
lacking the aptamer sequence was also used and one that
expressed the non-binding negative control sequence TR-D42.

(c) In vitro binding of aptamers and TR-aptamers

In vitro binding to the CD18cyt-peptide immobilized on a
sepharose matrix was confirmed for several aptamers (Figure
1B) using zonal elution quantitative affinity chromatography
assays as described in Dean et al., Affinity chromatography
a practical approach (1991), pp 169-174, IRL Press, Oxford,
UK. The binding affinity of monoclonal aptamers to the
immobilised CD18cyt-peptide was between 500 and 6000 nM
(Table 1). No binding of individual aptamers to un-
derivatised sepharose was observed. Importantly, correctly
terminated TR-aptamers were confirmed to exhibit the same in
vitro binding behaviour to the CD18cyt-peptide when
expressed in the context of the flanking stem-loop
structures. However, not terminated TR-aptamers with
additional 166 bases after the Terminator T ϕ derived from the
transfer TR-vector showed a 2-3 time reduction of binding
affinity for CD18cyt. Presumably this effect is due to the
interference of the additional sequence with the correct
folding of the aptamers. These results demonstrate that it
is possible to place the aptamers in the context of defined
functional sequences which add additional functionalities
such as increased stability. Correct termination is also
necessary as the functional nucleic acids exhibit reduced

ability to bind their target when too extensive additional sequences are present within the molecule.

(d) Cytoplasmic Expression of the functional nucleic acids

Cytoplasmic RNA expression relies on coinfection of cells with two recombinant vaccinia viruses (Fuerst and Moss, J. Mol. Biol. 206 (1989), 333-348). One virus, vaccinia-T7 (vT7), codes for the bacteriophage T7 RNA polymerase; the other, vTR-aptamer, encodes the sequence of the TR-aptamer and is derived from homologous recombination between vaccinia virus and the TR-aptamer vector. Vaccinia expression constructs (vTR-aptamer) were derived via recombination between the TR vector and wild type vaccinia virus (WR strain), high titer virus stocks were generated as described (Asubel et al. Current protocols in molecular biology (1987), Wiley, New York) and double infections with recombinant vaccinia viruses and a vaccinia virus encoding for T7-RNA polymerase (vT7) were carried out as described previously (Kolanus et al., Cell 86 (1996), 233-242). Briefly, 2×10^6 cells were collected by centrifugation, washed with PBS, resuspended in 300 μ l RPMI-medium. 100-150 μ l of each virus stock (MOI of ~ 10 pfu/cell, sonicated for 10 s) was added and incubated for 2 h at 37° C, followed by addition of 4 ml RPMI/10% fetal calf serum and additional incubation for between 1 and 7 h.

The course of RNA-expression after coinfection of a Jurkat E6 cell line with vT7 and vTR-D31 is shown in Figure 2C. Maximal amounts of RNA are reached 7 h post infection as quantified by dot-blot analysis using a DNA hybridisation probe complementary to the 3'-terminus of the aptamer expression cassette. Approximately 4×10^6 Jurkat E6 cells were infected with vaccinia viruses expressing TR-aptamer RNA and T7-RNA polymerase. RNA was isolated as described (Asubel et al. Current protocols in molecular biology (1987), Wiley, New York) and dot blotted onto a Hybond N membrane (NEN Du Pont) according to the manufacturers protocol. Hybridization of virus-expressed RNA was carried

out for 1 h by incubation of cellular RNA in pre-hybridization buffer (5 x SSC, 0.1% SDS, 5x Denhardt's reagent, 5% dextrane sulfate, 100 µg/ml tRNA) at 55° C, followed by incubation in the presence of 20 pmoles of radiolabeled probe MicTR1/28.1 (5'-CAAAAACCCCTCAAGACCCGTTTAGAG-3') in a total volume of 4.0 ml for 1 h. Dot-blots were then washed with ~20 ml 2x SSC, 0.1% SDS (2x), 1x SSC, 0.1% SDS (2x), and 0.5x SSC, 0.1% SDS (2x) at 55° C for 30 min, and quantified by phosphor imaging. Analysis of all TR transcripts *in vivo* at this peak revealed comparable aptamer RNA levels of roughly 10⁶ copies per cell, but only in the presence of vT7 (Figure 2D).

(e) Specificity of the TR-aptamers for endogenous CD18/β2 Integrin

Before investigating the biological effects of TR-aptamer expression it had to be confirmed that the aptamers are also able to specifically bind endogenous LFA-1. This was demonstrated by incubating radiolabelled aptamers transcribed *in vitro* from TR-aptamer vectors with crude cytoplasmic lysate from Jurkat E6 cells and subsequent gel electrophoresis under native conditions. 4·10⁷ Jurkat E6 cells were lysed in 400 µl of lysis buffer (PBS, 0.5% Triton X 100, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 mM PMSF, 1 mM MgCl₂) and incubated at 0° C for 20 min. Nuclei were removed by centrifugation at 3,000 g for 10 min and the supernatant was clarified by centrifugation at 10,000 g for 10 min. To 3 µl of this lysate, 2 µg of purified monoclonal antibody (MHM23, OKT3) or 2 µl ascites fluid (MEM170) were added and adjusted to 1x PBS in a total volume of 7 µl. After incubation at 0° C for 2-3 h, 5 µl of PBS containing 3 mM DTT, 1 mM MgCl₂, 30 U RNasin, 75-120 µM tRNA, 20% glycerol, 7.5 mg BSA were added and incubated at 0° C for 15 min. Following incubation, 30-60 fmol 5'-³²P-labeled TR-aptamer in 3 µl PBS, 1 mM MgCl₂, heated to 95° C for 30 s and cooled to 23° C for 10 min, was added and incubated at 23° C for 30 min. The lysate/aptamer/antibody mix was loaded onto a native 4.5% polyacrylamide gel (acrylamide/bis-acrylamide

60:1) containing 2% glycerol, and electrophoresed at 150 V for 2.5 h in 0.25 x TAE electrophoresis buffer.

As shown in Figure 3 in two representative experiments, this resulted in a pattern of shifted bands which were aptamer-specific. Aptamer TR-D20 shows a band-pattern with significantly reduced electrophoretic mobility (Figure 3A, lanes 2, 4) in comparison with the negative controls of lysate-free RNA (lane 1) and non-binding TR-D42 (lanes 3, 5) in the presence of a 10^4 -fold excess of tRNA as a non-specific competitor. To test whether the shifts were also LFA-1-specific, we performed a supershift analysis with two independent LFA-1-specific antibodies MHM23 and MEM170, directed against the extracellular domain of the β - and α -subunits of LFA-1, respectively, and the antibody OKT3 which recognises the ϵ -chain of the T-cell antigen receptor as a negative control. Addition of either MHM23 or MEM170 (lanes 6, 7) resulted in a supershifted band. In Figure 3B again both LFA-1-specific antibodies MHM23 and MEM170 (Figure 3B, lanes 3, 5) show the supershifted bands while no supershift was obtained with the non-cognate antibody OKT3 (lane 6). The experiments shown in lanes 4 and 7 establish that the aptamer TR-D20 has no direct affinity to either the specific antibody MHM23 or the non-specific antibody OKT3. When the gel-shift experiment shown in lane 2 was repeated in the presence of 2 mM unlabeled TR-D20 aptamer as a specific competitor, all radiolabeled TR-D20 RNA migrated at the level of unbound RNA (data not shown). Analogous results were obtained with TR-D28 and TR-D31 showing that the aptamers recognise endogenous CD18 in crude cell lysates with remarkable specificity.

(f) Determination of the binding site of the TR-aptamers on the intracellular domain of CD18

To define the binding site of the aptamers on the CD18cyt, gel-shift experiments were performed with three synthetic CD18cyt peptide fragments (Figure 5). Gel shifts employing synthetic biotinylated peptides were performed as follows: 5

μl of 3 nM radiolabeled aptamer RNA in PBS pH 7.4 was heated to 95° C for 30s and renatured for 10 min at 23° C. Biotinylated peptide was incubated with 2 mg/ml streptavidine in 20 mM Tris-HCl pH 7.4 in a total volume of 6 μl. To this solution a pre-mix was added to adjust the concentrations in the final 20 μl reaction volume to 1 x PBS, 1 mM DTT, 1 mM MgCl₂, 7.5% glycerol, 2.5 mg/ml BSA, 40 μM tRNA, 2U/ml RNasin, and pre-incubated for 20 min at 23° C. The aptamer solution (5 μl) was added, incubated for 20 min at 23° C, and electrophoresed on a native 6% polyacrylamide gel as described above.

No binding to the carboxy terminal half of CD18cyt (peptide A23) was observed with any of the aptamers, but TR-D20, TR-D28, and, more weakly, TR-D31 all bound to both the N-terminal peptide B16 and the middle portion C17. B16 and C17 overlap by 8 amino acids including a cluster of three basic arginine residues. That this highly positively charged cluster was selected by the aptamers as a binding site on CD18cyt is not surprising because an aptamer is a highly negatively charged molecule. As expected, the negative control aptamer TR-D42 exhibited no binding to any of the three fragments used confirming that the peptide-fragment recognition is aptamer-specific.

(g) Effects of the intracellular TR-aptamers on the integrin mediated cellular adhesion to intercellular adhesion molecule 1 (ICAM1)

To test whether the cytoplasmic expression of CD18-specific aptamers has an effect on the biological activity of this integrin adhesion assays with Jurkat E6 cells or with peripheral blood mononuclear cells (PBMC) were performed (Coligan et al., (1994) Current protocols in immunology (John Wiley & Sons, New York). The cells were infected with vaccinia viruses encoding CD18-specific aptamers or various control sequences, and binding of these cells to an important ligand of LFA-1, the ICAM-1 molecule, was assessed *in vitro*. This system has an additional level of control

since it was known that TR-aptamer expression is absolutely dependent upon co-infection of cells with a virus expressing T7 polymerase (Figure 2D). The ICAM-1-Rg fusion protein was expressed in COS cells, purified from culture supernatants by Protein A-Sepharose, eluted, resuspended in PBS and coated onto plastic dishes as described (Kolanus et al., Cell 86 (1996), 233-242). $2 \cdot 10^6$ Jurkat or PBM cells were infected with recombinant vaccinia viruses and incubated for 4-8 h at 37°C. After centrifugation, cells were resuspended in RPMI medium and incubated for 5 min at 37°C with or without the addition of 40 ng/ml PMA. Cells were subsequently allowed to adhere to ICAM-1-Rg coated dishes at 37°C for 30 min and the bound fraction was determined with the aid of an ocular reticle. Figure 4A shows that infected Jurkat E6 cells displayed considerable background adhesion to ICAM-1, but this was nonetheless superinducible by phorbol-12-myristate-13-acetate (PMA), a well known promoter of LFA-1-mediated leukocyte adhesion (Lub et al., Immunol. Today 16 (1995), 479-483). Notably, whereas the TR without insert and single infections with wild-type vaccinia virus (wt), vT7, or vTR-D20 had almost no effect, expression of the TR-D20 aptamer in Jurkat cells resulted in an almost complete block of inducible adhesion.

The reproducibility and extension of the method to other systems was tested by performing similar studies with human peripheral blood mononuclear cells (PBMC). PBMC, which are normally difficult to transfect, proved to be superb targets for transient gene expression mediated by recombinant vaccinia viruses. A flow cytometric analysis of vaccinia expressed control proteins, sub-domain derivatives of cytohesin-1, clearly shows that Ig-fusion proteins with the cytohesin-1 plextrin homology (PH) domain (cIg-PH) and the PH+c-domains (cIg-PH+c), or the isolated Ig portion itself (cIg) were all expressed at equally high levels in human PBMC (Figure 4B). These controls, chosen because the cytoplasmic cytohesin-1 has previously been shown to be an important regulator of $\beta 2$ integrin adhesive function (Kolanus et al., Cell 86 (1996), 233-242; Nagel et al., J. Biol.

Chem. 273 (1998), 14853-14861; Nagel et al., Mol. Biol. Cell 9 (1998), 1981-1994), and the aptamer viruses were tested in adhesion assays with these cells as described above. The cytohesin-1 PH+c-domains (cIg-PH+c) had a strongly suppressive effect on PMA-inducible adhesion whereas both the isolated PH domain (cIg-PH) which shows considerably reduced phospholipid-binding and membrane association (Nagel et al., Mol. Biol. Cell 9 (1998), 1981-1994), or the control protein had no effect (Figure 4C). These results are in accordance with published data obtained with Jurkat cells (Nagel et al., J. Biol. Chem. 273 (1998), 14853-14861). Most importantly, Figure 4C also shows that TR-D20 aptamer mediated reduction of PMA-inducible adhesion in PBMC is similar to that observed with cIg-PH+c, whereas the negative control sequence TR-D42 had no effect on $\beta 2$ integrin-mediated adhesion to ICAM-1. Taken together, these results indicate that the adhesive function of LFA-1 was specifically reduced in Jurkat E6- and PBM cells by the cytoplasmic expression of an aptamer selected *in vitro* to bind the cytoplasmic portion of the receptor (Figure 4A,C) (Lub et al., Immunol. Today 16 (1995), 479-483).

Example 2: Functional target identification of intracellular components involved in the induction of the IL-2 Promotor

Example 2 demonstrates a positive selection scheme incorporating negative selection proofreading to isolate intracellular targets which participate in signal transduction events that lead to the induction of genes under the control of the interleukin 2 promotor (IL-2 P) in T cells. After stimulation of the T-cell receptor (TCR), genes are induced by a signal transduction cascade under the control of the interleukin-2 promotor. Participating in the signal transduction cascade are, among others, proteins such as protein kinase C, calcineurin, NFkB etc. (see: Abbas et al. (Eds.), Cellular and molecular immunology 2nd ed. (1994), p. 162, W.B. Saunders Company). The block of calcineurin by immuno-suppressing pharmaceuticals such as cyclosporin A, FK 506 can, for example, turn off the

induction of the promotor and thus the production of cellular cytokines. This is used in the clinic for preventing rejection of organ transplants. The IL-2 promotor is an excellent model system with calcineurin as an example for the clinically relevant functional targets or key-molecules that should be identified.

a) Phenotypic selection

(1) Construction of the IL-2-Promotor reporter cell line

A reporter-gene that encodes for a toxin (Herpes simplex virus thymidine kinase, HSV TK) (Fife et al., Gene Ther. 5 (1998), 614-620) under the control of the IL-2 promotor is cloned in a vector containing a selectable marker (aminoglycoside phosphotransferase, neo) (Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327-341) that allows the selection of cells with the genes of interest inserted in their chromosomes by detoxification of aminoglycosid antibiotics (neomycin, G418) ((Fig.1, plasmid pP1). The IL-2 promotor will be induced when introduced into T cell lines upon stimulation with phorbol esters or via the T cell receptor (TCR) and the herpes simplex virus thymidine kinase (HSV-TK) will be expressed. Addition of ganciclovir results in the formation of toxic compounds (e.g. Kanai et al., Cancer Res. 56 (1996), 1341-5) and subsequent cell death.

After transfection of a T cell line (Jurkat E6 cells) and culturing in medium (RPMI, 10% FCS, Gentamycin 50 µg/ml) containing 0.1-1.5 mg/ml neomycin (depending on the minimal lethal concentration for the cell line used) and isolation of single stable transfectants the cells were analyzed for the presence and expression of the reporter gene via standard methods (e.g. Northern Hybridisation, Western blotting).

(2) Construction of the RNA expression vectors

A ssDNA library is synthesized , e.g. via standard solid phase synthesis, where the region of random sequence is flanked by fixed sequences that serve as primer annealing

sites and contain endonuclease restriction sites for cloning in the expression vectors. dsDNA for example is enzymatically synthesized using PCR or Klenow protocols. This corresponds to the standard protocols for generating nucleic acid libraries for *in vitro* selection experiments (see e.g. Klug et al., Proc.Natl.Acad.Sci 94 (1997), 6676-81; Abelson, Methods in Enzymology 267 (1996), pp 275-81, Academic Press, San Diego)

The library of random sequence genes was cloned under the control of a RNA polymerase III promotor (Pol III P) which allows high level RNA expression (Fig.6, plasmid pP2). Additionally the library is flanked by conserved sequences that are able to form a stem similar to the adenoviral VAI RNA terminal stem (vai random vai) (Furtado et al., J. Virol. 63 (1989), 3423-3434). The resulting RNA transcripts therefor consist of the sequence : 5'-GGGCACUCUCCGUG-N40-AACGGGGGAGCGCUCCUUUU-3', whereby N40 represents forty contiguous random positions with equal stoichiometry of all four bases (A,C,G,U). It was previously shown that those constructs are exported very efficiently and from the nucleus to the cytoplasm of eukaryotic cells (Bertrand et al., Program & Abstracts RNA '98: The third annual meeting of the RNA Society 26.-31.5.1998, University of Wisconsin, Madison). In this context most of the RNA library is exported into the cytoplasm where most of the targets are located which participate in the induction of the IL-2 promotor.

The plasmid (Fig 6, plasmid pP2) additionally contains a selectable marker (hygromycin) (The antibiotic hygromycin and resistance marker genes are commercially available and described, e.g. Invitrogen BV, Groningen, Netherlands) and elements for episomal replication to ensure maintainance of the plasmid (OriP, EBNA1-protein gene) (Yates et al., Nature. 313 (1985), 812-815; Chittenden et al., J. Virol. 63 (1989), 3016-3025). This could be necessary if the selection process proceeds for a longer time period. The library plasmid is transfected into the reporter cell line and the time course, maximum RNA library expression and RNA localisation are

determined using standard techniques such as Northern blots and in situ hybridisations. In a typical transfection experiment 10^7 - 10^8 cells are transfected using electroporation or lipofection. RNA samples prepared from the transfected cells were isolated and analyzed according to the methods described in Example 1 ((d) Cytoplasmic Expression of the functional nucleic acids) and probed with a radiolabelled antisense oligonucleotide (5'-AAAAGGAGCGCTCCCCCGTT-3'). The study revealed expression levels of roughly 5×10^5 RNA copies/cell.

(3) Selection:

After transfection and expression of the RNA library the reporter cells (stable transfected Jurkat E6 cells) are now selected for the phenotype of inhibited IL-2 promotor induction. The cells are stimulated with phorbol-12-myristate-13-acetate (PMA) (50 ng/ml) (Kolanus et al., Cell 86 (1996), 233-242) and incubated in the presence of ganciclovir (Obaru et al., Hum. Gene Ther. 7 (1996), 2203-2208). Only those cells expressing a functional nucleic acid from the library that inhibits the induction or expression of the HSV-TK gene (see Figure 8 blocking protein X of the schematically drawn signal transduction cascade X, Y, Z) will survive this selection. Other cells will die (Fig. 7). In order to allow the enrichment of cells the stringency of the selection can be varied if necessary employing different concentrations of ganciclovir, different incubation periods or repeated cycles of stimulation and incubation with the drug. The remaining cells can be harvested and the plasmids carrying the gene for the inhibiting RNA molecule transfected for amplification into *E.coli* using the "hirt supernatant" method. The cells are lysed in 0.4ml 0.6% SDS, 10 mM EDTA, 1'-20'. The viscous mixture is pipetted into a microfuge tube: After addition of 0.1ml 5M NaCl and mixing, the samples are incubated 5 hrs on ice. After spinning 4 min, carefully removing supernatant, phenol extraction, addition of 10 micrograms linear polyacr acids. Second, all functional nucleic acids can be eliminated that just act by

inhibiting the toxin and not act on the signal transduction directly.

(c) Identification of functional cellular targets

(1) Construction of the vectors for the functional target identification:

The main functional elements of the suitable plasmid are depicted in Figure 9.

plasmid pF1:

- Promotor elements:

CMV P: human cytomegalovirus immediate early promotor. Example of a constitutive promotor element that is active in a wide variety of cell types. Depending on cell lines, application, etc. other frequently used promotors or combinations of promoter elements (E.g. SV 40 early promotor, Rous sarcoma virus immediate early promotor etc.) can be used (Spector et al., Cells: A Laboratory Manual (1998), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 67.2-67.3).

IL-2 P: IL-2 Promotor

- Episomal replication:

The Epstein Barr Virus (EBV) origin of replication (OriP) and nuclear antigen (EBNA-1) allow the high copy episomal (extra chromosomal) replication and maintenance in primate and canine cell lines (Yates et al., Nature 313 (1985), 812-815; Chittenden et al., J. Virol. 63 (1989), 3016-3025). This is an optional element but allows the selection to proceed over a longer time period if necessary.

- cDNA library:

The cDNA library in this example is derived from T cells. The library is used to select for the complementation of the inhibited signal transduction cascade. Overexpression of the factor that is inhibited by the functional nucleic acid

should encompass the block in signalling and the IL-2 promotor be induced again in cells harbouring the target gene of interest. Construction and expression of cDNA libraries are well known to a person skilled in the art.

- fNS-hsv thymidine kinase:

This construct allows the identification of the target gene product through the specificity of the nucleic acid-protein interaction. The gene of the functional nucleic acid (fNS) is cloned in the 5' untranslated leader of the herpes simplex virus thymidine kinase. Werstuck and Green could show that constructs which harbour aptamer sequences in the 5' untranslated leader are very useful for controlling the expression of genes (*Science* 282 (1998), 296-298). Upon binding of the aptamer ligand (functional nucleic acid) the expression of the reporter HSV TK is inhibited presumably through blocking the translation.

- Selectable marker:

neo: commonly used resistance gene (aminoglycosid phosphotransferase) to select for cells harbouring the transfected plasmids via detoxification of aminoglycosid antibiotics (Southern and Berg, *J. Mol. Appl. Genet.* 1 (1982), 327-341).

Plasmid pF2:

-Promotor elements (:

See pF1. Additionally RNA Polymerase III (Pol III P) promotor-VAI/fNS/VAI construct. This construct permits as described in the functional selection step the high level transcription and export of the functional nucleic acid in the cytoplasm.

- Episomal replication: .See pF1.

- Selectable marker:

Resistance against hygromycin can be used to coselect for cells that harbor two plasmids (e.g. additionally a plasmid with the neo resistance)

- mIg: This is a surface marker that allows the affinity selection of transformed cells. In this example the constant CH2 and CH3 domains of human IgG1 expressed as transmembrane version (mIg) that lead to their presentation on the cell surface. The cells can be isolated for example using immobilized commercially available antibodies (e.g. AffiniPure goat anti-humanIgG, Fcy fragment, dianova, Hamburg). Fusionproteins with the constant CH2 and CH3 domains of human Ig molecules are a well established. Technique for the expression of proteins (Kolanus et al., Cell 86 (1996), 233-242).

(2) The selection process:

After transformation of plasmids pF1 and pF2 in a T cell line (e.g. Jurkat E6 cells) the cells are coselected in the presence of neomycin and hygromycin. The Pol III transcripts of the functional nucleic acid (fNS) will block the signaltransduction cascade (schematically Proteins X, Y, Z) by binding to the endogenous protein X. Overexpression of most proteins (schematically protein M) from the cDNA library will have no effect on the blocking of the signal transduction cascade and the IL-2 promotor constructs will not be induced (Fig. 10). The expression of the cognate target (protein X, Fig. 11) for the functional nucleic acid (fNS) from the cDNA library leads to the complementation of the signalling to the IL-2 promotor by bypassing the block of the functional nucleic acid. The reporter genes for mIg and HSV TK are turned on. The mIg is used to isolate those cells where the signal transduction pathway is rescued via affinity isolation. Because the levels of protein X inside the T-cells are held at saturating concentrations using high level expression promoters protein X also binds to the mRNA of HSV TK and prevents its expression by blocking its translation. The cells will survive culture in the presence of ganciclovir. If necessary several rounds of gene induction and selection can be repeated and finally the

plasmids with the cDNA genes be isolated for example using the "hirt supernatant" method.

The elegance of this system is its dual read out. The target gene is identified via two different properties. It's gene product has to be able to functionally rescue the signal transduction in a homologous system for the affinity selection of the cells via the mIg. Furthermore, the target protein protects the cells from being killed because it specifically binds to the functional nucleic acid (aptamer) RNA cloned into the 5'-UTR of the HSV-TK mRNA which is induced by the target protein itself. This approach makes use of alternate conformations of RNA-aptamers which can be controlled in dependence of the presence or absence of the cognate aptamer ligand. This is common knowledge because detailed structural investigations of many aptamer/ligand complexes by multi-dimensional NMR spectroscopy have established that RNA/ligand complex formation is always accompanied by extensive conformational changes and conformational stabilization of the binding RNA-receptor only in the presence of the cognate ligand (Patel et al., J. Mol. Biol. 272 (1997), 645-664; Patel, Curr. Opin. Chem. Biol. 1 (1997), 32-46; Fan et al., J. Mol. Biol. 258 (1996), 480-500; Dieckmann et al., RNA 2 (1996), 628-640; Yang et al., Science 272 (1996), 1343-1347). Furthermore, Werstuck and Green [Science 282 (1998), 296-298] established that the translation of a gene can be prevented if an aptamer sequence is inserted into the untranslated region of an expression plasmid in the presence of the aptamer's cognate ligand, but proceeds normally in its absence. The need to be functional and specific in two independent systems reduces the danger of obtaining false positive cDNA clones. Clones that activate the IL-2 promoter by deregulation of the signalling (e.g. protein N acting on protein Z, fig. 12) should fail to bind specifically to the fNS in the HSV-TK mRNA and to block its expression. Therefore they will not survive the selection because of the ganciclovir mediated cell killing. Finally the system provides specific modulators that can be easily used in the target validation

described herein to elucidate the complex protein networks inside cells.

Table 1

aptamer	K _{ds} (μM)
D20	1.08
TR D20	1.37
TR-D20 RT	2.25
D28	0.55
TR D28	0.53
TR D28-RT	1.82
D31	6.14
TR D31	3.88
TR D31 RT	>13.7

Table 1: K_{ds} (μM) of CD18cyt binding aptamers to the cytoplasmatic domain of CD18. TR indicates correctly terminated TR-aptamers in the TR-vector expression cassette context. RT : readthrough of not correctly terminated TR-aptamers with additional 166 nucleotides

CLAIMS:

1. A method for the identification of an intramer capable of binding to and modifying the function of a functional intracellular target, comprising:
 - a) preparing a candidate mixture of nucleic acids;
 - b) contacting the candidate mixture of nucleic acids with the intracellular target or a part thereof;
 - c) selecting and isolating nucleic acids having an increased affinity to said target relative to the candidate mixture;
 - d) reverse transcribing, if the candidate mixture comprises RNAs and amplifying the nucleic acids obtained in step c);
 - e) optionally repeating steps b) to d);
 - f) isolating and sequencing the clones (intramers) obtained in step e); and
 - g) testing whether the expression product of the insert of the clone obtained in step f) binds to and effects the function of the intracellular target in vivo.
2. The method of claim 1, wherein step g) is carried out by using a cytoplasmic expression system.
3. The method of claim 1 or 2 further comprising
 - h) mapping of the binding site of the intramer to said target.
4. A method for the identification of a functional intracellular target which is associated with a particular phenotype and the corresponding intramer capable of binding to and modifying the function of said target, comprising:
 - a) preparing a candidate mixture of nucleic acids;
 - b) cloning the candidate mixture of nucleic acids under the control of a suitable promotor in a vector optionally containing a selectable marker;

- c) introducing the vector obtained in step b) into a reporter cell line allowing positive or negative phenotypic selection;
 - d) selecting cells with an altered phenotype; and
 - e) determining the sequence of the nucleic acid inserted in the vector of step b) (intramer) and the compound which it binds to.
5. The method of any one of claims 1 to 4, wherein said candidate mixture of nucleic acids comprises single stranded nucleic acids.
 6. The method of claim 5, wherein the single stranded nucleic acid is a RNA.
 7. The method of any one of claims 1 to 6, wherein the functional intracellular target is an integrin.
 8. The method of any one of claims 4 to 7, wherein the reporter cell line of step c) allows negative selection.
 9. The method of claim 8, wherein the reporter cell line contains a vector comprising a selectable marker and a reporter gene encoding a toxin under the control of an inducible promotor and wherein only cells will survive expressing the vector of step b) which expresses a nucleic acid (intramer) inhibiting a compound which is required for the activation of the promotor controlling the toxin gene, and wherein in step d) the surviving cells are selected.
 10. The method of any one of claims 4 to 9 wherein the candidate mixture of nucleic acids of the vector of step b) is under the control of a Pol III promotor.
 11. The method of claim 9 or 10 wherein the toxin gene is HSV-thymidine-kinase.
 12. The method of any one of claims 9 to 11 wherein the promotor controlling the toxin gene is a IL-2 promotor.

13. A host cell containing one or more vectors having the candidate mixture recited in claims 1 to 12 as insertions and/or the vector of claim 9.
14. A pharmaceutical composition containing a nucleic acid (intramer) which is capable of modifying the function of the functional intracellular target which it binds to and which is identified according to the methods of any one of claims 1 to 12, optionally in combination with a suitable pharmaceutical carrier.
15. A pharmaceutical composition containing a compound which is capable of modifying the function of the functional intracellular target identified by the method of any one of claims 1 to 12.
16. Use of the nucleic acid identified by the method of any one of claims 1 to 12 or a compound which is capable of modifying the functional intracellular target identified by the method of any one of claims 1 to 12 for the preparation of a medicament for gene therapy.
17. Kit useful for the diagnosis of diseases associated with abnormal concentrations and/or modifications of the intracellular target which a nucleic acid identified by the method of any one of claims 1 to 12 binds to comprising the nucleic acid (intramer) identified by the method of any one of claims 1 to 11 or an antibody having the same or a similar target specificity.

ABSTRACT

THE
HONORABLE
MEMBERS OF THE
LEGISLATIVE ASSEMBLY
OF THE PROVINCE OF ONTARIO

AND
THE
JUDGES OF THE
SUPREME COURT OF JUSTICE
OF CANADA

IN
PARLIAMENTARY SESSION,
AT TORONTO,

ON
THURSDAY, MAY 10, 1917.

PRESENTED BY
MR. JAMES H. COOPER,
SOLICITOR GENERAL.

AND
BY MR. J. M. GIBSON,
ATTORNEY GENERAL.

PRINTED AT THE
GOVERNMENT PRINTING OFFICE,
TORONTO.

1917.

A 5'-TC [T7-prmator] GGGCGCTAAGTCCTCGCTCA-N40-ACGGCGGACTCGGATCCT-3'

DNA-pool

H₂N-KALIIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTVMNPKFAES-COOH

CD18-cyt (46-mer peptide)

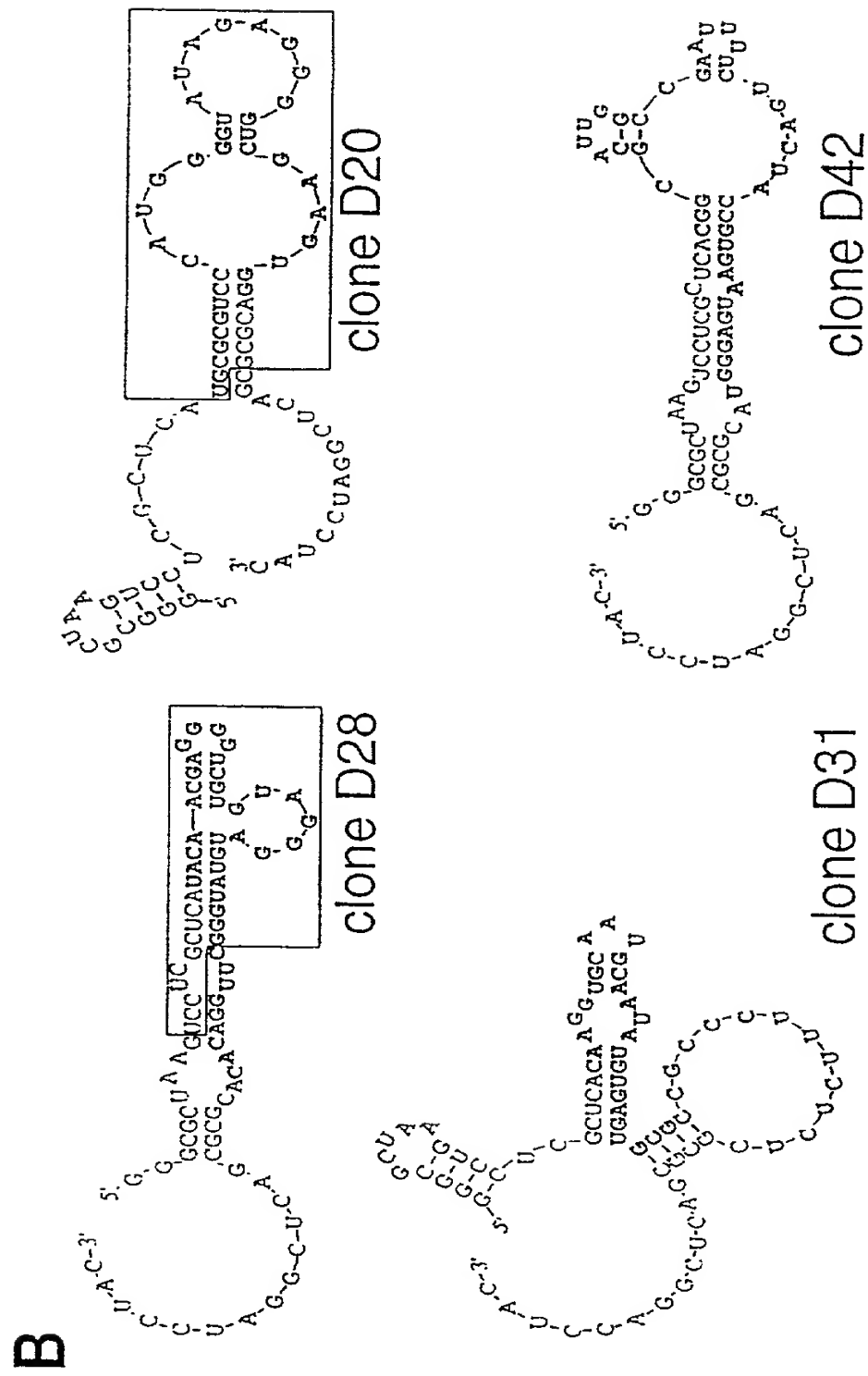


Figure 1

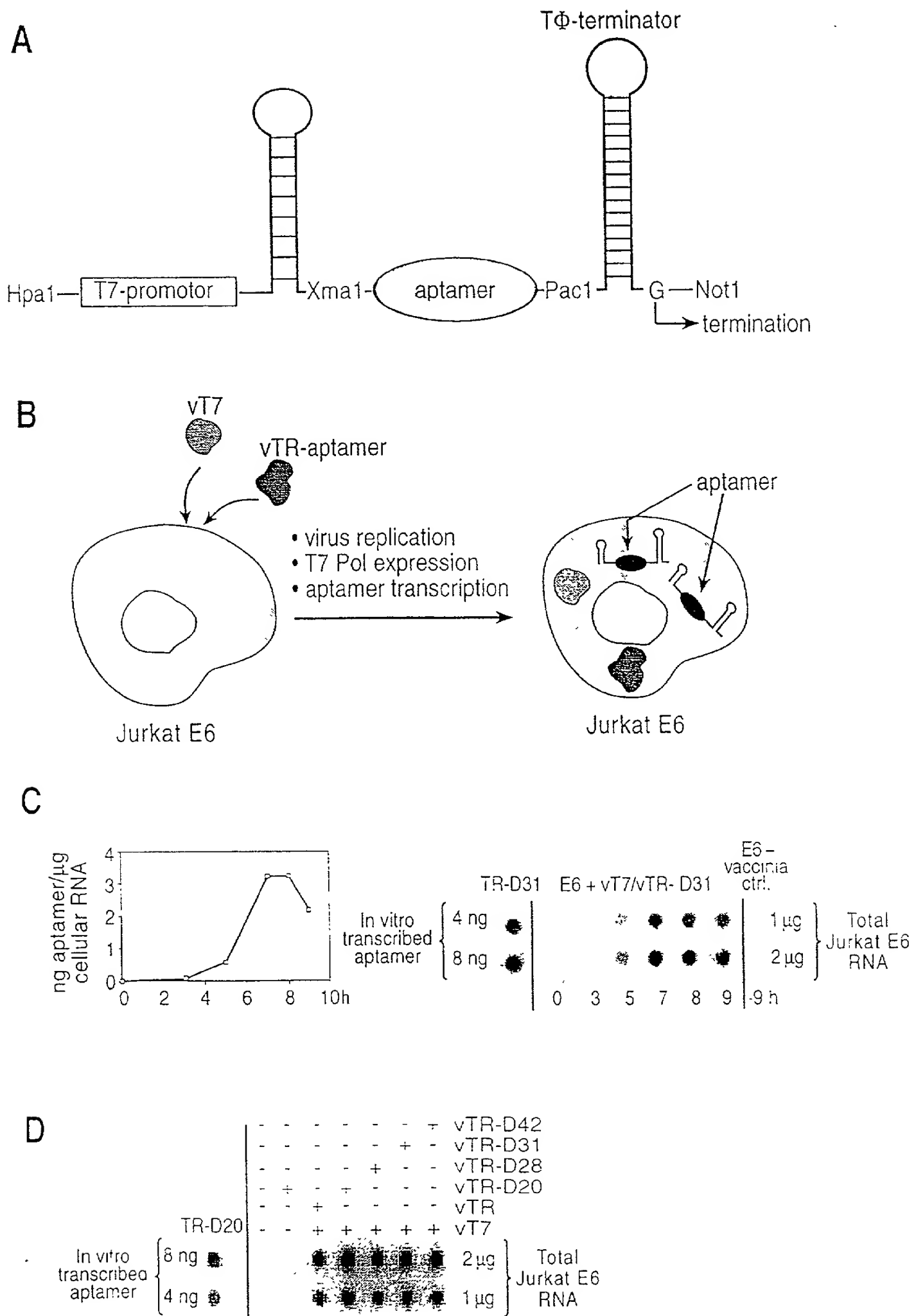


Figure 2

3/12

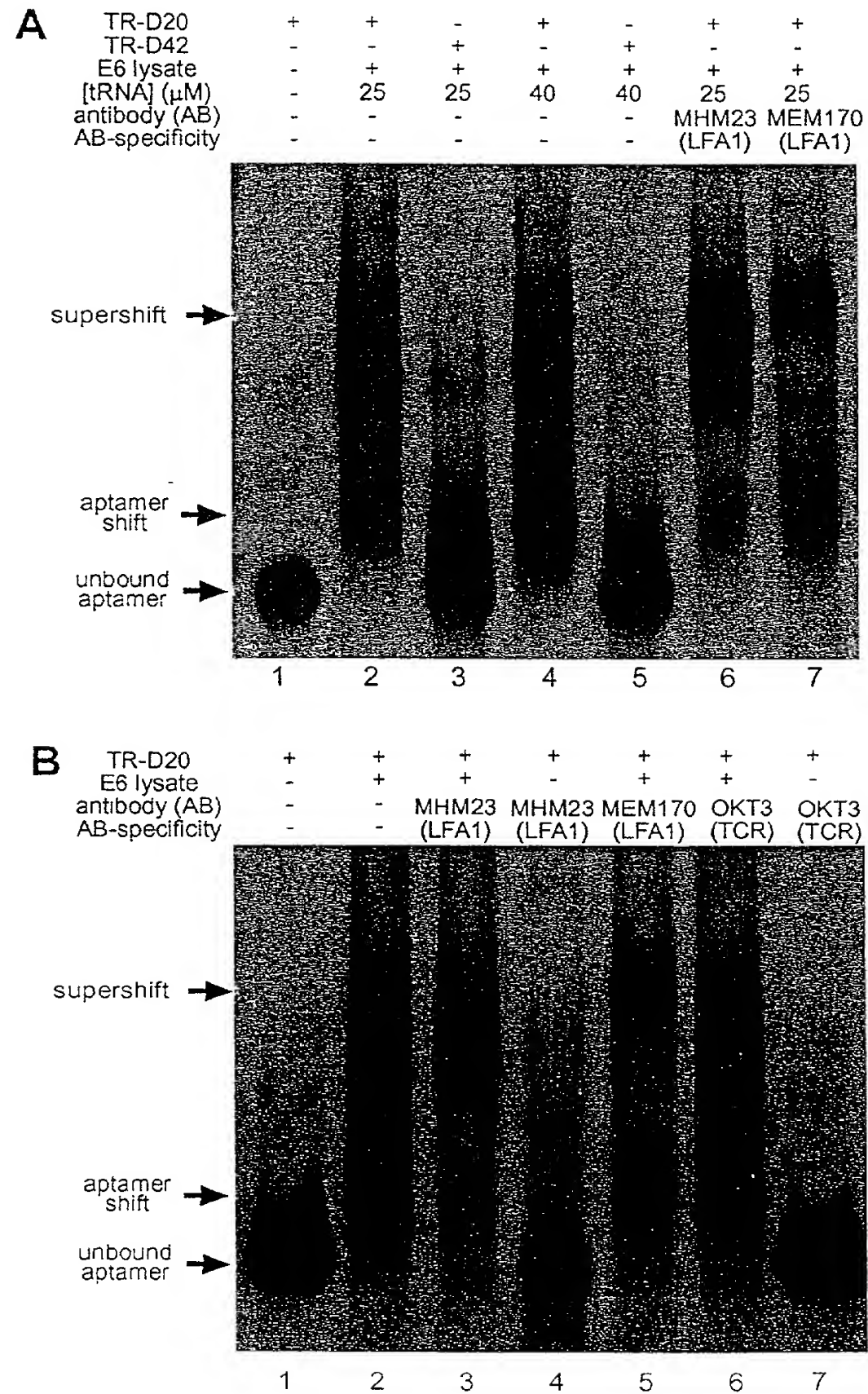


Figure 3.

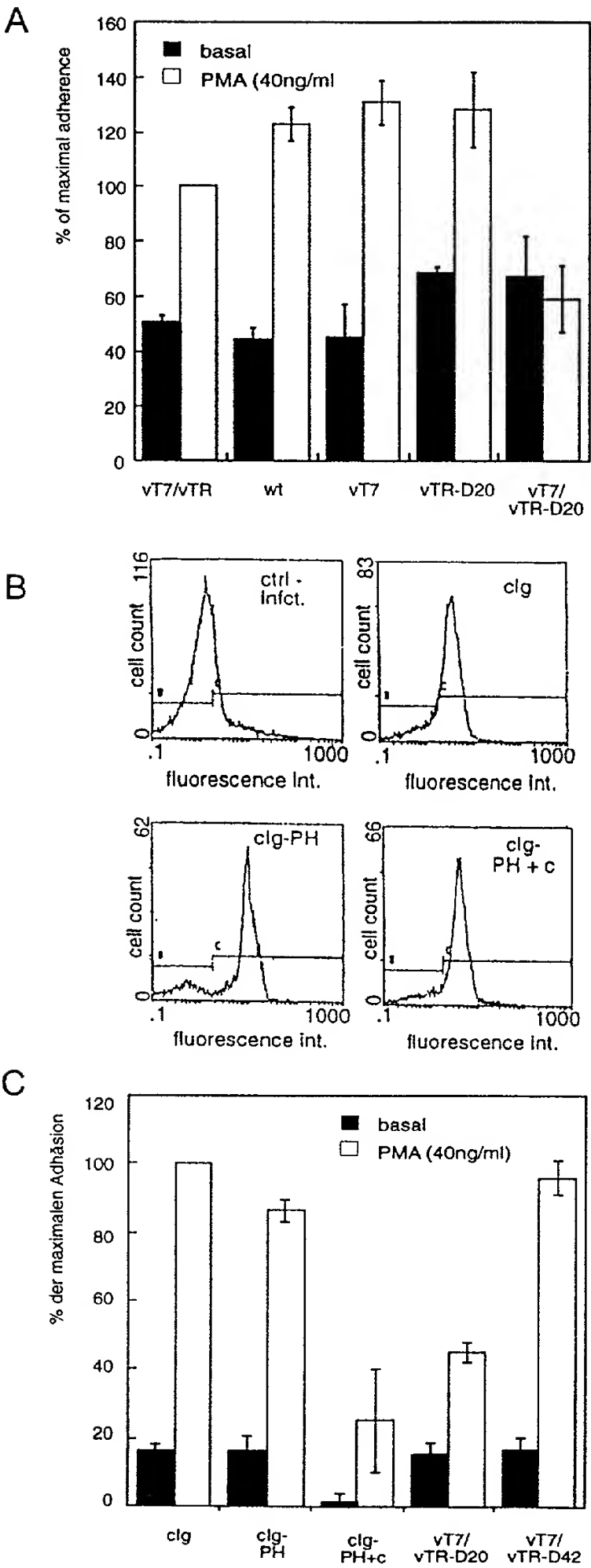


Figure 4

5/12

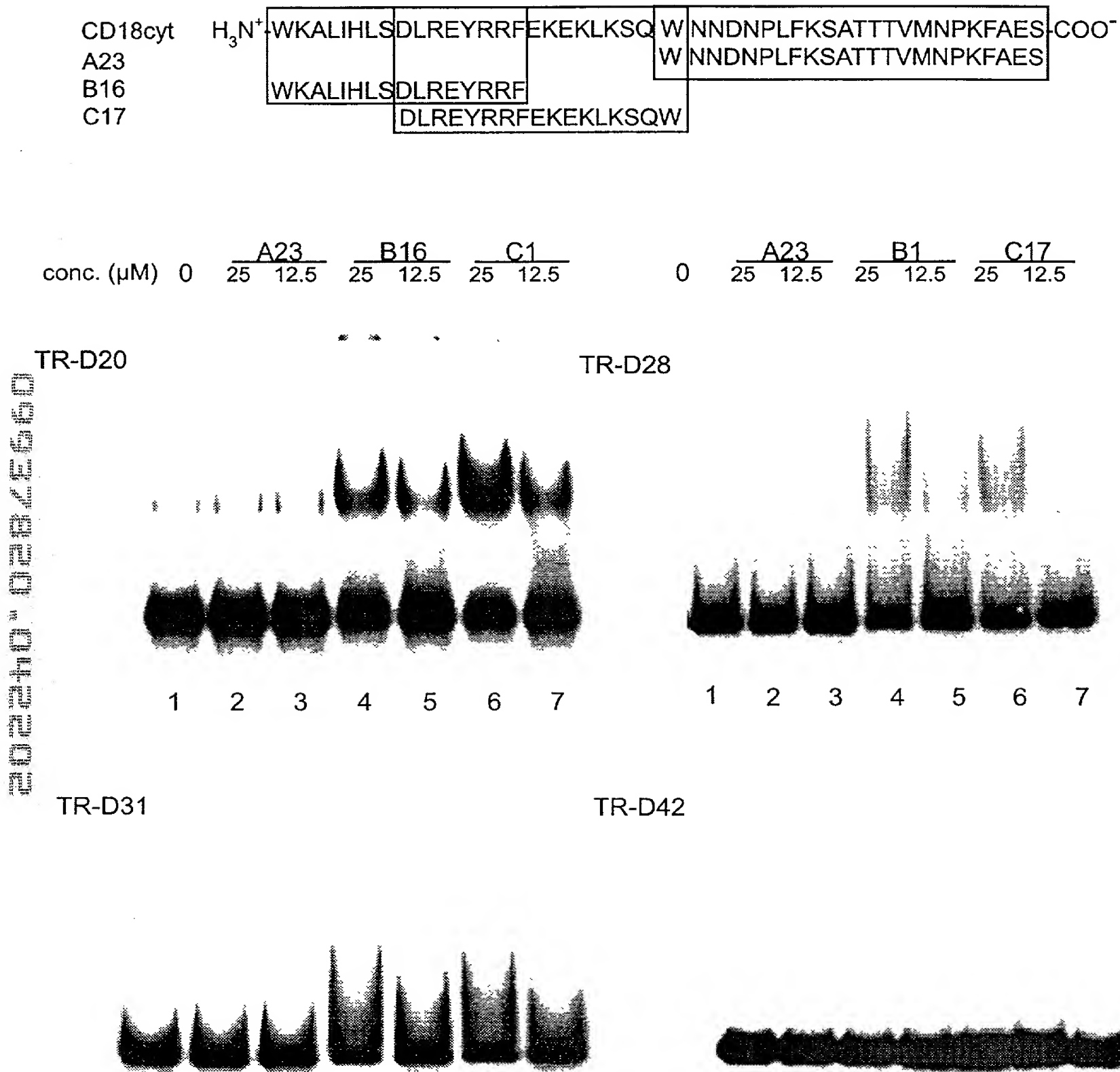
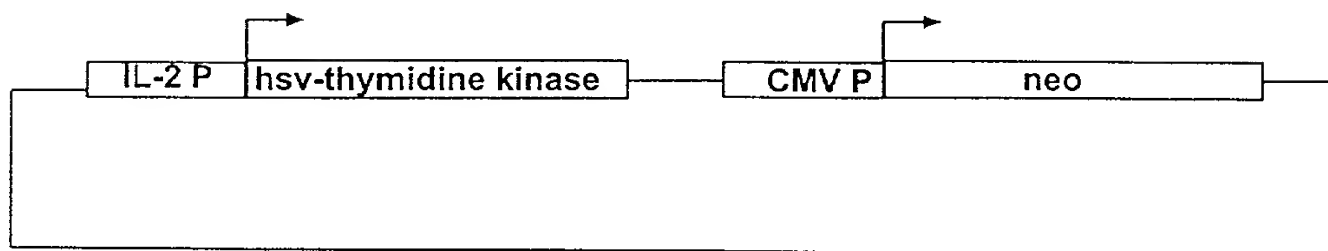


Figure 5

6/12

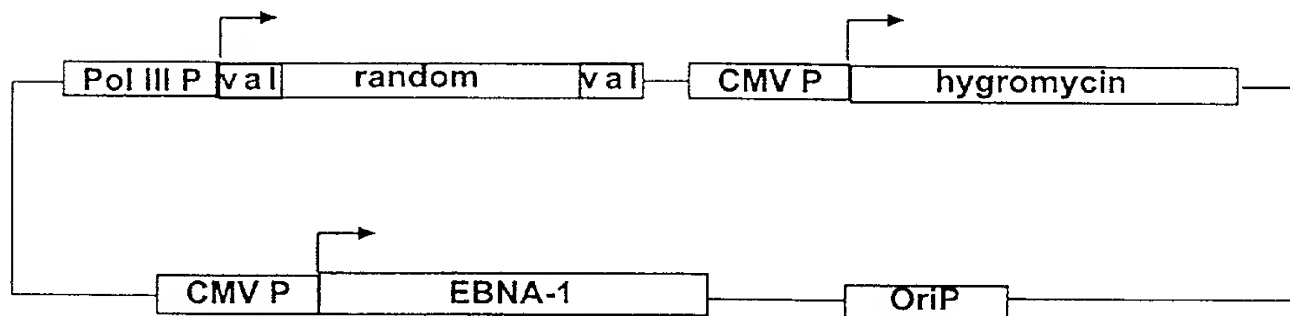
Vectors for the phenotypic selection against IL-2 promotor induction

A



plasmid pP1

B



plasmid pP2

Figure 6

Figure 7.

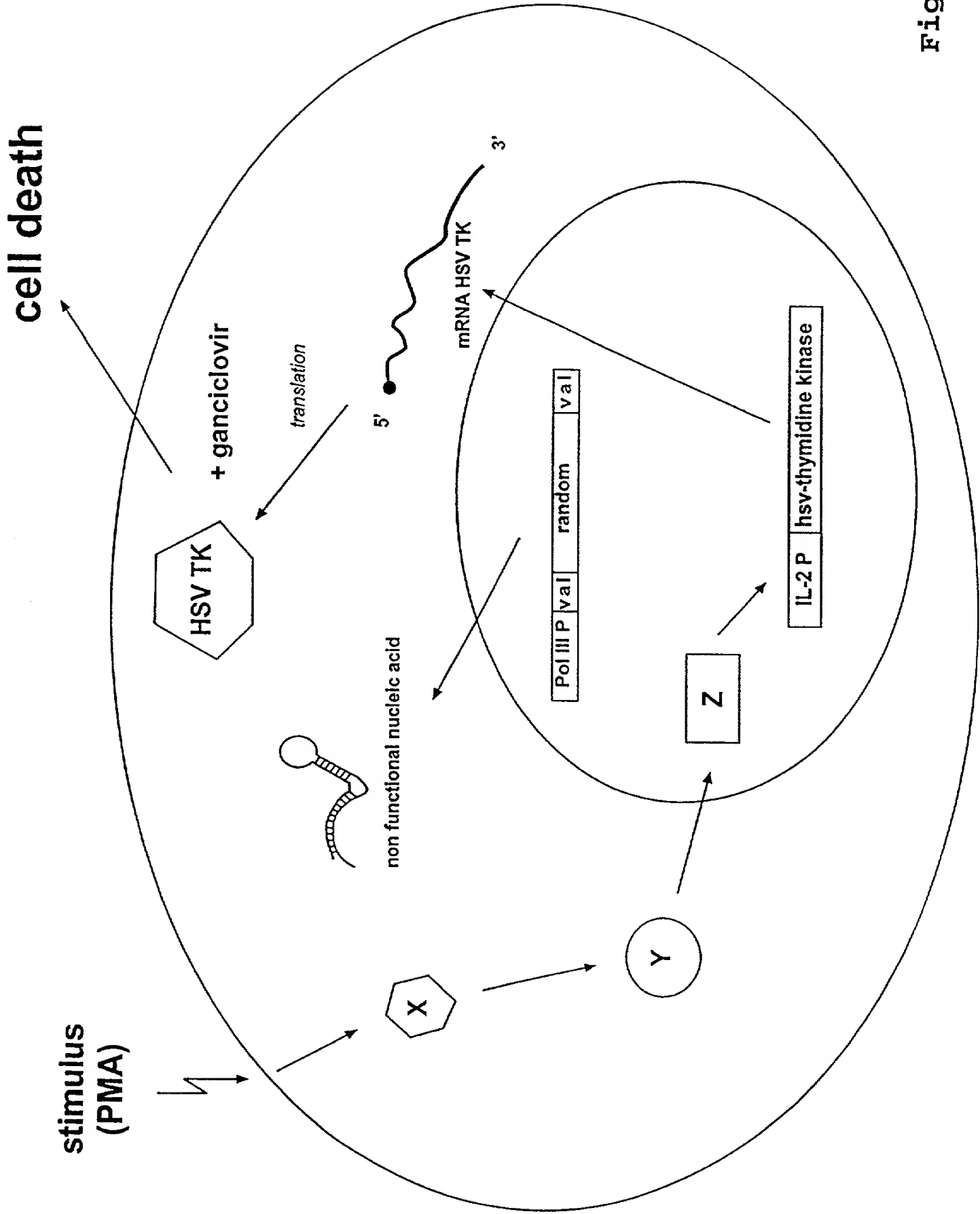
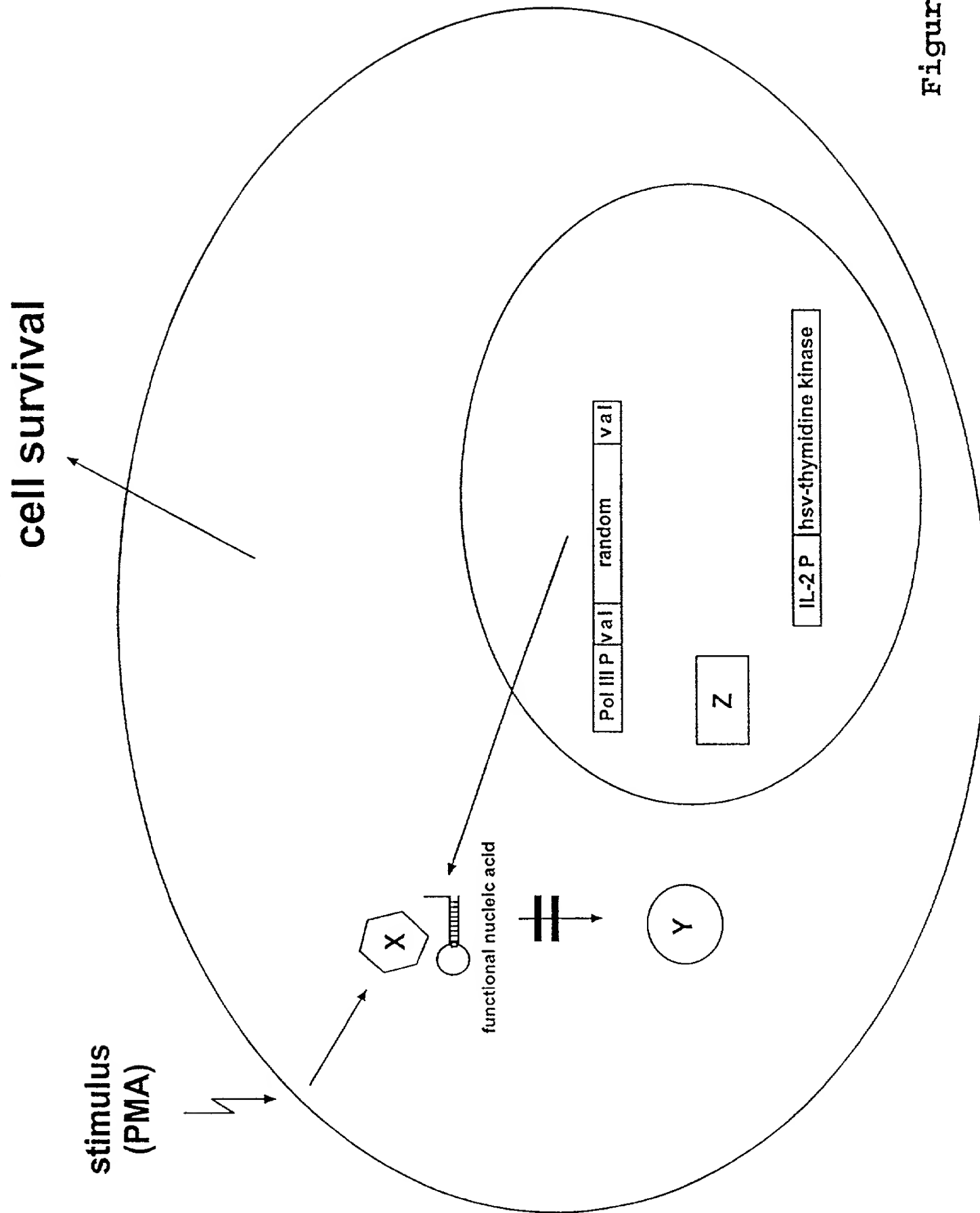
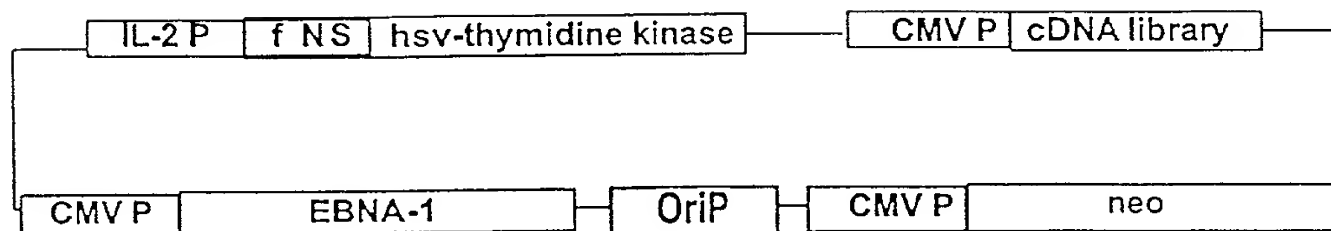


Figure 8



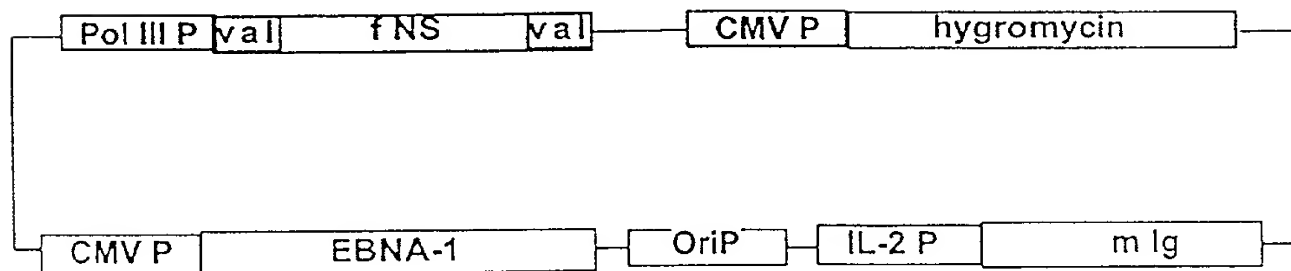
Vectors for the functional target identification

A



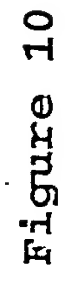
plasmid pF1

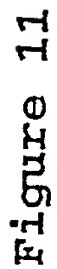
B



plasmid pF2

Figure 9





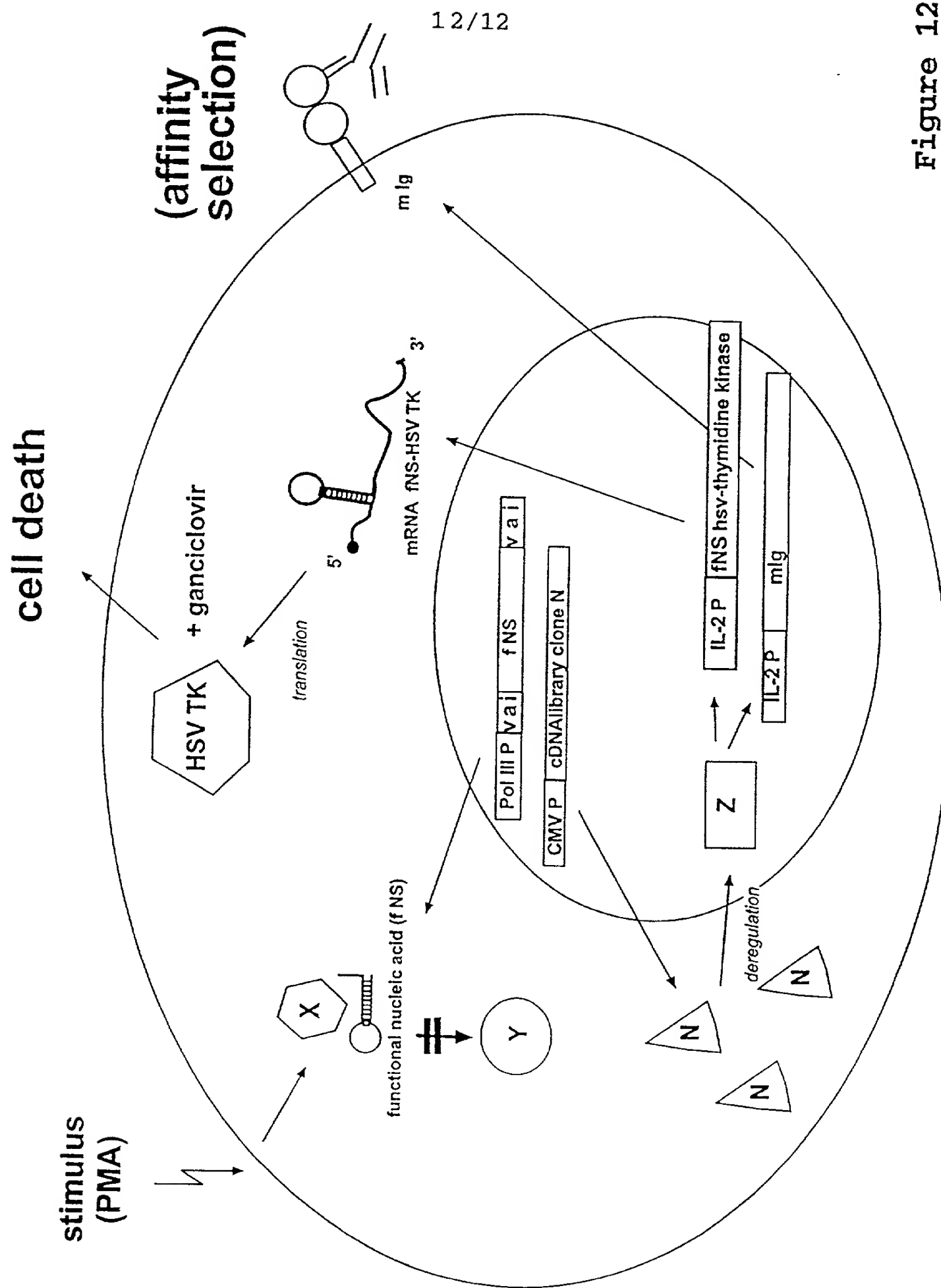


Figure 12



Express Mail Label No.
Docket No. **P1561USA**

Page 1 of 4

Declaration and Power of Attorney For Patent Application English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Methods for the Identification and Validation of Functional Intracellular Targets with
Intramers or *in vivo* Selection

the specification of which

(check one)

☐ is attached hereto

☒ was filed on March 28, 2000 as United States Application No. PCT/EP00/02727 or PCT International Application Number _____ and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

99106453.6

EP

3/29/99

☐

(Number)

(Country)

(Day/Month/Year Filed)

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Page 3 of 4

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

9 — Phillip G. Hampton, II Reg. No. 30,642
Richard W. Young Reg. No. 30,706
Brian C. Rupp Reg. No. 35,665
Thomas A. Belush Reg. No. 37,090
Lisa V. Mueller Reg. No. 38,978
Vernon W. Francissen Reg. No. 41,762
Michael M. Geoffrey Reg. No. 41,775
Connie B. Berg Reg. No. 46,548
Michael R. Osterhoff Reg. No. 48,973

of GARDNER CARTON & DOUGLAS, 321 North Clark Street, Suite 3400, Chicago, Illinois 60610-4795

Send Correspondence to:

Lisa V. Mueller
GARDNER CARTON & DOUGLAS
321 North Clark Street
Suite 3300
Chicago, IL 60610-4795

Direct Telephone Calls to: (name and telephone number)

Lisa V. Mueller
(312) 245-8732

Full name of sole or first inventor: Michael Blind

Sole or first inventor's signature [Signature]

Date

27.3.02

Residence Seinsheimstrasse 11, D-81245 Munchen, Germany DEX

Citizenship German

Post Office Address : Seinsheimstrasse 11, D-81245 Munchen, Germany

200 Full name of second inventor: Michael Farniglok

Second inventor's signature [Signature]

Date

28.3.02

Residence Leonardusstrasse 26, D-53175 Bonn, Germany DEX

Citizenship German

Post Office Address

27/03/2002 15:21 00951556413
S.: BORMANN & LOOSEN;

08951558413; ,

27-MAR-02 14:18;

SEITE 9/9

300

Full name of third inventor: Waldemar Kolanus

Second inventor's signature

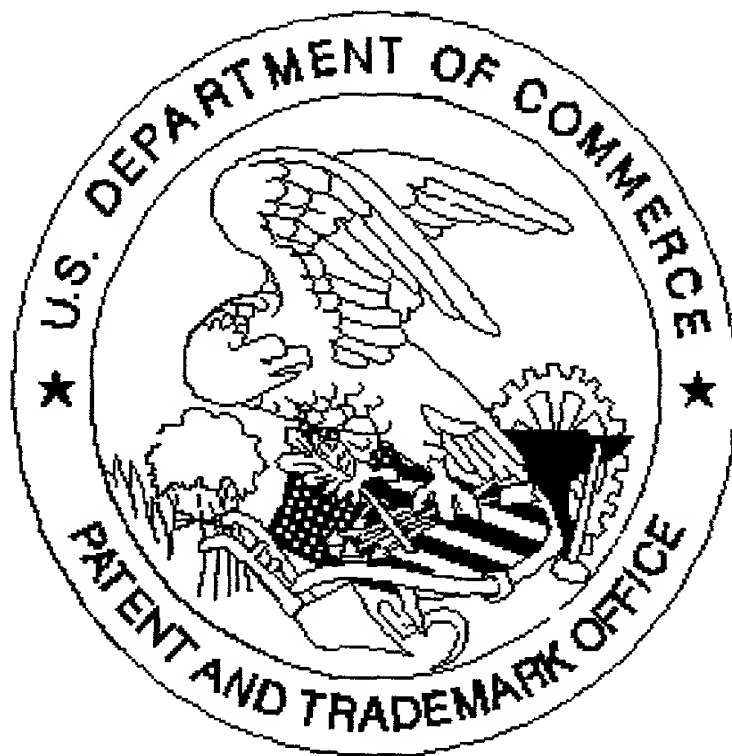
Date 3/27/02.

Residence Gosswinstrasse 7, D-81245 Munchen, Germany

Citizenship German

151111
 151112
 151113
 151114
 151115
 151116
 151117
 151118
 151119
 151120
 151121
 151122
 151123
 151124
 151125
 151126
 151127
 151128
 151129
 151130
 151131
 151132
 151133
 151134
 151135
 151136
 151137
 151138
 151139
 151140
 151141
 151142
 151143
 151144
 151145
 151146
 151147
 151148
 151149
 151150
 151151
 151152
 151153
 151154
 151155
 151156
 151157
 151158
 151159
 151160
 151161
 151162
 151163
 151164
 151165
 151166
 151167
 151168
 151169
 151170
 151171
 151172
 151173
 151174
 151175
 151176
 151177
 151178
 151179
 151180
 151181
 151182
 151183
 151184
 151185
 151186
 151187
 151188
 151189
 151190
 151191
 151192
 151193
 151194
 151195
 151196
 151197
 151198
 151199
 151200
 151201
 151202
 151203
 151204
 151205
 151206
 151207
 151208
 151209
 151210
 151211
 151212
 151213
 151214
 151215
 151216
 151217
 151218
 151219
 151220
 151221
 151222
 151223
 151224
 151225
 151226
 151227
 151228
 151229
 151230
 151231
 151232
 151233
 151234
 151235
 151236
 151237
 151238
 151239
 151240
 151241
 151242
 151243
 151244
 151245
 151246
 151247
 151248
 151249
 151250
 151251
 151252
 151253
 151254
 151255
 151256
 151257
 151258
 151259
 151260
 151261
 151262
 151263
 151264
 151265
 151266
 151267
 151268
 151269
 151270
 151271
 151272
 151273
 151274
 151275
 151276
 151277
 151278
 151279
 151280
 151281
 151282
 151283
 151284
 151285
 151286
 151287
 151288
 151289
 151290
 151291
 151292
 151293
 151294
 151295
 151296
 151297
 151298
 151299
 151300
 151301
 151302
 151303
 151304
 151305
 151306
 151307
 151308
 151309
 151310
 151311
 151312
 151313
 151314
 151315
 151316
 151317
 151318
 151319
 151320
 151321
 151322
 151323
 151324
 151325
 151326
 151327
 151328
 151329
 151330
 151331
 151332
 151333
 151334
 151335
 151336
 151337
 151338
 151339
 151340
 151341
 151342
 151343
 151344
 151345
 151346
 151347
 151348
 151349
 151350
 151351
 151352
 151353
 151354
 151355
 151356
 151357
 151358
 151359
 151360
 151361
 151362
 151363
 151364
 151365
 151366
 151367
 151368
 151369
 151370
 151371
 151372
 151373
 151374
 151375
 151376
 151377
 151378
 151379
 151380
 151381
 151382
 151383
 151384
 151385
 151386
 151387
 151388
 151389
 151390
 151391
 151392
 151393
 151394
 151395
 151396
 151397
 151398
 151399
 151400
 151401
 151402
 151403
 151404
 151405
 151406
 151407
 151408
 151409
 151410
 151411
 151412
 151413
 151414
 151415
 151416
 151417
 151418
 151419
 151420
 151421
 151422
 151423
 151424
 151425
 151426
 151427
 151428
 151429
 151430
 151431
 151432
 151433
 151434
 151435
 151436
 151437
 151438
 151439
 151440
 151441
 151442
 151443
 151444
 151445
 151446
 151447
 151448
 151449
 151450
 151451
 151452
 151453
 151454
 151455
 151456
 151457
 151458
 151459
 151460
 151461
 151462
 151463
 151464
 151465
 151466
 151467
 151468
 151469
 151470
 151471
 151472
 151473
 151474
 151475
 151476
 151477
 151478
 151479
 151480
 151481
 151482

United States Patent & Trademark Office
Office of Initial Patent Examination -- Scanning Division



Application deficiencies found during scanning:

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☒ Scanned copy is best available. *Figure 3 is dark.*